UNIVERSIDADE DE LISBOA

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THE EFFECTS OF MACRONUTRIENT COMPOSITION OF THE LARVAL DIET ON LIFE HISTORY TRAITS AND PIGMENTATION IN *DROSOPHILA VIRILIS*

Marisa Almeida Rodrigues

DISSERTAÇÃO

MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO

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Abstract

One of the main contributions for an animal's life success is an optimal nutrition. Macronutrients, such as proteins and carbohydrates, are essential for organism development, determining for example, the size of the body and or reproductive capacity. Different animals use macronutrients differently. To achieve the necessary requirements, generalist species use a wide range of substrates, whereas specialist species are specialised in one type of substrate. In general, animals balance their food intake to achieve nutritional optima, referred as intake target. Uncovering an animal's intake target requires solving the problem of balancing multiple and changing nutrient needs in a variable nutritional environment. To address this we can study the nutritional geometry framework of an animal. Foraging decisions can then be described within this nutrient space, however these decisions may bring consequences for the animals' development. In this thesis, we measured the influence of unbalanced larval diets on life history traits, such as survival, developmental time, body size, ovariole number and pupal case pigmentation. We also addressed this by analysing the consequences on foraging behaviour. We found that *Drosophila virilis* maximises life history traits at a high protein to carbohydrate ratio and pupal pigmentation changes by increasing the content of protein on larval diet. However, larvae do not regulate their intake to maximise any trait responses.

Since intake target changes over developmental time and evolutionary time, we expected to see differences between generalists and specialists species. We used our previous data from *Drosophila melanogaster*, a generalist species, to compare with the results from this thesis. We saw differences, whereas *D. virilis* seem to be more tolerant to high proteins than *D. melanogaster* but less tolerant to high carbohydrates content. Depending on their feeding strategies, species will always differ in nutritional requirements and foraging strategies in unbalance nutritional environments.

Keywords: nutritional geometry, intake target, life history traits, foraging behaviour, pigmentation.

Resumo

A qualidade nutricional da comida é essencial ao desenvolvimento dos organismos. Sabemos que os macronutrientes, entre eles as proteínas e os hidratos de carbono, são importantes para formação e manutenção de tecidos ou fornece uma das principais fontes de energia aos processos metabólicos, respectivamente. A alimentação é o único meio pelo qual os organismos conseguem adquirir os nutrientes de que necessitam, sendo que as suas necessidades não são sempre as mesmas. Dependendo da espécie, cada nutriente é necessário em quantidades distintas, assim como a relação entre nutrientes é variável..

Os animais regulam e tomam decisões relativamente à comida ingerida. Estratégias de comportamento relativas à alimentação foram desenvolvidas consoante as necessidades de cada espécie. Dois grupos podem ser definidos, relativamente a estas estratégias: espécies generalistas, que são espécies que usam uma gama variada de substratos para satisfazer as suas necessidades nutricionais; e espécies especialistas que satisfazem as suas necessidades nutricionais utilizando um número muito restrito de substratos. Os substratos sofrem alterações nutricionais ao longo do tempo. Embora o ambiente seja responsável por parte dessas alterações, microrganismos desempenham um papel fundamental e, por isso, nem sempre existe um substrato com a composição nutricional ideal que se mantenha por muito tempo. Devido ao carácter nutricional instável de cada substrato, os animais ponderam quais as escolhas possíveis de forma a atingir os valores nutricionais ótimos para o seu desenvolvimento, que se define como alvo nutricional.

O alvo nutricional pode ser encontrado usando o método desenvolvido por Steve J. Simpson e David Raubenheimer em 1990, o modelo de geometria nutricional. Este método permite criar um espaço nutricional com base num gradiente de concentrações de dois nutrientes e avaliar as decisões dos animais nesse espaço. Este método permite-nos descrever como os animais se comportam em três cenários diferentes. Primeiro, têm à sua disposição uma dieta equilibrada, e comem até atingirem os níveis nutricionais ótimos. Segundo, podem ter à disposição duas dietas, ambas desequilibradas, o que resulta numa ingestão alternada de ambas as dietas para que se possa atingir os níveis nutricionais ideias. Terceiro, apenas está disponível uma única dieta, que é desequilibrada. Neste caso, existem duas decisões possíveis para que os animais atingirem os níveis ótimos. Uma alternativa é que um dos nutrientes se revela mais importante, e a quantidade ingerida é regulada de forma e atingir os níveis ótimos apenas para essa nutriente, ingerindo o segundo em excesso ou em défice. A outra alternativa é a ingestão de níveis intermédios para ambos os nutrientes. Para descobrir o alvo nutricional é necessário explorar como é que os animais tomam estas decisões, como é que preenchem as suas necessidades nutricionais num ambiente nutricionalmente variável. Este método já deu provas do seu potencial, desde de

mamíferos, como humanos e ratos, a invertebrados, como gafanhotos, escaravelhos, aranhas e moscas, onde foi verificado que todos estes animais regulam a ingestão de nutrientes (Simpson and Raubenheimer, 2005; Mayntx *et al.*, 2005).

O mesmo alvo nutricional pode não ser mantido ao longo da vida do animal, sofrendo mudanças dependendo da espécie. No entanto, também se altera consoante o estado fisiológico e estadio do ciclo de vida. Por exemplo, quando a mosca da fruta, *Ceratitis capitata* está perto da metamorfose, o seu alvo nutricional deixa de ser maioritariamente proteico e passa a conter alto teor de hidratos de carbono, que irão providenciar energia para a fase que precede a metamorfose (Zucoloto, 1987). O alvo nutricional também se altera quando as fêmeas de *Drosophila melanogaster* acasalam (Ribeiro and Dickson, 2010). Após acasalarem, a produção de ovos é estimulada e as fêmeas passam a consumir uma dieta mais rica em proteínas do que as que ainda são virgens. O consumo de dietas proteínas em gafanhotos é alterado para um menor consumo de proteína quando deixa de haver crescimento de tecidos (Raubenheimer and Simpson 1999).

As espécies generalistas e as especialistas podem ter o mesmo alvo nutricional mas desenvolveram diferentes estratégias para o atingir. As suas necessidades nutricionais podem ser diferentes, como é o caso da *Schistocerca gergaria*, uma espécie generalistas, que mostra maior tolerância a elevados níveis de proteína do que a espécie especialista, *Locusta migratoria* (Raubenheimer and Simpson, 2003). A mesma situação foi encontrada em espécies de *Lepidoptera* generalistas e especialistas (Lee *et al*., 2002 and 2003).

Nesta tese, decidimos primeiro avaliar com este método como as características que têm um papel na *fitness* dos animais é afetada pelos macronutrientes, proteínas e hidratos de carbono, na espécie especialista, *Drosophila virilis*. Esta espécie tem como principal fonte de alimento a seiva das árvores. Por fim, comparamos a resposta de uma espécie generalista (*D. melanogaster*), descrita anteriormente e da espécie especialista (*D. virilis*) descrita nesta tese.

As características avaliadas são conhecidas por serem influenciadas por diferentes ambientes nutricionais. O tempo de desenvolvimento da fase larval é afectado pela nutrição, tal como a sobrevivência. Também estudámos a influência de dietas desequilibradas no tamanho do corpo de adulto, pesando as pupas antes do adulto emergir. Observámos que quanto mais pobre em proteína é a dieta mais pequenos são os indivíduos. O número de filamentos que constituem os ovários, chamados de ovaríolos, está diretamente relacionado com o número de ovos que uma fêmea irá pôr, ao longo da vida, e varia com a qualidade da dieta. Sendo *Drosophila* um organismo holometábolo, ou seja, sofre uma total metamorfose antes da fase adulta. Uma vez que é nesta fase que todos os tecidos e órgão se preparam para dar origem às estruturas e órgãos do adulto, todas as características descritas neste estudo são analisadas na fase larval. Depois da metamorfose os indivíduos param o seu crescimento, ou seja o tamanho do adulto é definido na fase de larva. Também o número de ovaríolos é determinado na fase de larva.

Para conseguirmos desvendar a influência dos macronutrientes, fornecemos aos indivíduos, várias dietas que diferiam entre si pelo rácio entre proteínas e hidratos de carbono (rácio P:C) e também no seu teor calórico. Com o nosso espaço nutricional definido, analisámos quando indivíduos formaram pupa, quando tempo demoraram até formarem pupa, qual o seu peso antes do adulto emergir e o no caso das fêmeas quantos ovaríolos têm em ambos os ovários.

Os nossos resultados mostraram que é nas dietas com um rácio entre proteínas e hidratos de carbono elevado que os indivíduos maximizaram a sua sobrevivência, tamanho do corpo e número de ovaríolos, e minimizam o tempo de desenvolvimento. Enquanto que no caso de *D. melanogaster*, as diferentes características foram maximizadas por diferentes dietas.

Decidimos em seguida analisar o como as larvas de *D. virilis* reagem quando confrontadas com um ambiente de duas dietas desequilibradas. Que decisões irão tomar? Esta parte do processo baseou-se em analisarmos o comportamentos tanto das larvas como das fêmeas adultas.

No caso das larvas, analisámos as decisões que estes indivíduos tomaram para satisfazerem os seus requisitos nutricionais. O nosso procedimento passou por usar larvas no terceiro estádio larvar e dar-lhes duas opções de dietas. Verificámos que, de facto, as larvas regulam a quantidade de ambas as dietas ingeridas de modo a alcançar valores específicos de proteína e hidratos de carbono, no entanto estes não correspondem aos valores que optimizam as características acima referidas. Quando comparado com os dados de *D. Melanogaster*, as larvas regularam a ingestão dos nutrientes de forma a minimizar o tempo de desenvolvimento.

Relativamente ás fêmeas adultas, analisámos tanto o seu comportamento de alimentação, como na escolha de local para oviposição. Quando as fêmeas chegaram ao pico de fertilidade, fornecíamos a machos e fêmeas três dietas nutricionalmente desequilibradas. Fizemos contagem de quantas fêmeas comeram de cada dieta e do número de ovos postos em cada dieta. Os nosso resultados mostraram que as fêmeas não fizeram nenhuma escolha sobre qual das dietas ingerir. Adicionalmente, não encontramos nenhuma escolha de preferência para pôr os ovos. O oposto tinha sido visto em *D. melanogaster*, onde as fêmeas fizeram escolhas sob qual a dieta a ingerir, elevado teor de proteína, e em qual colocar os ovos, elevado teor de hidratos de carbono.

Durante o protocolo do modelo de geometria nutricional, deparamo-nos com diferenças na pigmentação dos casulos de pupa. Fizemos, então, novamente este protocolo, de forma a quantificar as diferenças de pigmentação de acordo com as diferentes dietas. As larvas desenvolveram-se nas mesmas dietas usadas anteriormente, e após o adulto emergir as pupas vazia eram retiradas e fotografadas. Utilizando Mathematica, calculámos um valor RGB da coloração de cada pupa. Os nossos resultados mostram um gradiente de pigmentação que varia com a quantidade de proteína na dieta. Quanto mais proteína, mais escuras são as pupas.

Podemos assim concluir que os macronutrientes, de facto, influenciam tanto o desenvolvimento dos animais como o seu comportamento. Também podemos observar que os macronutrientes afetam de forma variada cada espécie.

Palavras-chave: modelo de geometria nutricional, alvo nutricional, nutrição, comportamento, pigmentação.

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1. INTRODUCTION

The nutritional environment of an organism has profound impacts on its development, life history, behaviour and evolution. Species are adapted to different nutritional niches, which change with time, season and with interactions with microorganisms. Generalist species use a wider range of food sources, whereas specialist species are more adept at using a specific, preferred substrate. Their adaptation to different substrate breadths cause generalist and specialist species to differ in their developmental characters, life history traits and foraging patterns.

In a previous study conducted in a generalist species of fruit fly, the common laboratory species *Drosophila melanogaster*, we explored how life history characters respond to variation in larval diet. Here, we extend this previous work to understand how life history traits, morphological characters and foraging strategies respond to variation in larval nutrition in a species that specializes in feeding on tree sap, *Drosophila virilis*.

To these ends, we examined the responses of four life history characters and a developmental trait, the pigmentation of the pupal case, to the protein, carbohydrate and caloric content of the larval diet. In addition, we examined how larval and adult feeding and oviposition choices relate to the developmental and life history responses to larval diet. Our aim was to qualitatively compare the responses in these characters between *Drosophila* species to assess how the nutritional biology of the organism changes with differences in niche breadth.

1.1 STRATEGIES TO COPE WITH COMPLEX NUTRITIONAL ENVIRONMENTS

Food is the only channel through which animals can get the nutrients that they cannot themselves produce. The macronutrients, vitamins and minerals found in food are crucial for an organism's development and survival, but they also differ in their functions. For example, while proteins are essential for tissue formation and maintenance during development, reproduction and somatic maintenance, carbohydrates and lipids provide essential sources of energy for metabolic processes in addition to their structural functions in the cell. Thus, rather than serving simply as fuel in the form of calories, the correct balance of macronutrients from food is necessary to ensure correct cellular functions.

Animals balance their food intake to reach their desired combination of macronutrients, their so-called intake target (Simpson and Raubenheimer, 1993). Uncovering an animal's intake target requires exploring how an animal solves the problem of balancing multiple and changing nutrient needs in a variable nutritional environment. In practical terms, this can be found using the nutritional geometry framework, a method developed by Steve J. Simpson and David Raubenheimer in early 1990's that co-varies the concentration of two nutrients across a range of values to generate a nutrient space and assessing how animals make foraging decisions within

this space. The nutritional geometry framework describes how animals make foraging decisions using three different scenarios.

Scenario 1 – Nutrient regulation on a balanced substrate. In this case the animal has available a source of food already containing its desired balance of macronutrients. To reach its intake target, the animal only needs to regulate the amount of food it consumes (Figure 1.1-A) (Raubenheimer and Simpson 1999).

Scenario 2 – Nutrient regulation on an unbalanced substrate. Here, the substrate available is nutritionally unbalanced (Figure 1.1-B). In this case by eating this substrate the animal will never reach its intake target, and is forced to make decisions about how to trade-off between paucity versus excess of the two nutrients.

Scenario 3 – Nutrient regulation between two unbalanced foods. In this scenario, animals can choose between two nutritionally-unbalanced substrates (Figure 1.1-C). Here the animal can reach its intake target by alternately ingesting both substrates.

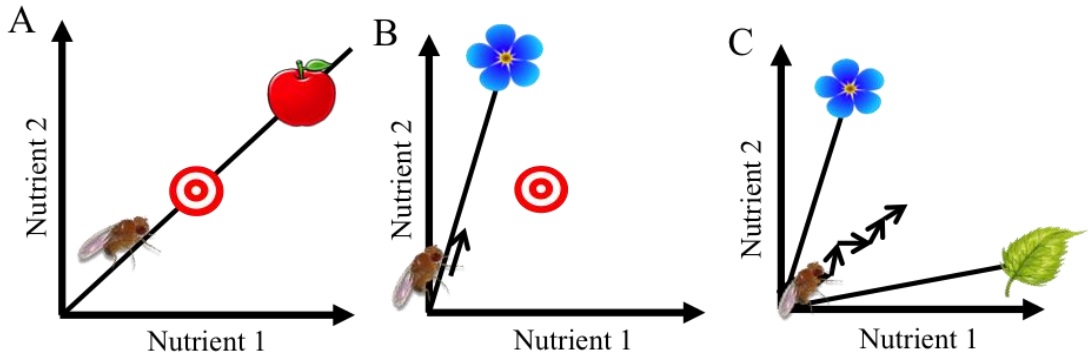

Figure 1.1 – Representation of the nutrient space and the balance of nutrient is described by a linear trajectory. (A) When animals feed on balance food and reach the intake target. (B) When animals can only feed on an unbalance food, consequently animals do not reaching the intake target. (C) When exists two unbalance food available, switching between both foods, animals can reach the intake target. Figure redrawn from Raubenheimer and Simpson, 1999.

Exploring foraging strategies using these three scenarios provides substantial information about how animals make foraging decisions. Either balanced or complementary diets can be used to uncover the animal's target intake. In unbalanced diets, we can explore the rules of compromise when the intake target cannot be reached. If nutrient 1 is more important, animals will regulate their ingestion to reach that optimal amount, even if it means that they have to ingest an excessive amount of the nutrient 2. Alternatively, animals can choose to minimize the excess and deficit, or nutritional error, of both nutrients (Simpson and Raubenheimer, 1993).

We can obtain limited information regarding the rules of compromise for a single unbalanced food. A complete exploration of these rules implies observing intake strategies over several unbalanced diets. In this way, we can represent the foraging strategy an animal uses by the shape of its intake array relative to the animal's actual

intake target (Simpson and Raubenheimer, 2012.). Based on simple decisions like the above, animals will follow different rules, dependently of what is more important for them:

1. They can eat the same volume of food without regulating for nutrient intake;

2. They eat to reach intake target levels for one of the nutrients, without regulating the intake of the second nutrient;

3. They can try to reach the intake target for both nutrients, even if this means that one nutrient is ingested in excess (Figure 1.2-A);

4. They eat until they reach the target levels for one of the nutrients, then they will stop eating, which will result in a under consumption of the other nutrient;

5. They eat until the sum of both nutrients ingested is equal to their sum at the intake target (Figure 1.2-B);

6.They eat until they reach the closest geometrical point to the intake target, thereby minimizing nutritional error (Figure 1.2-C).

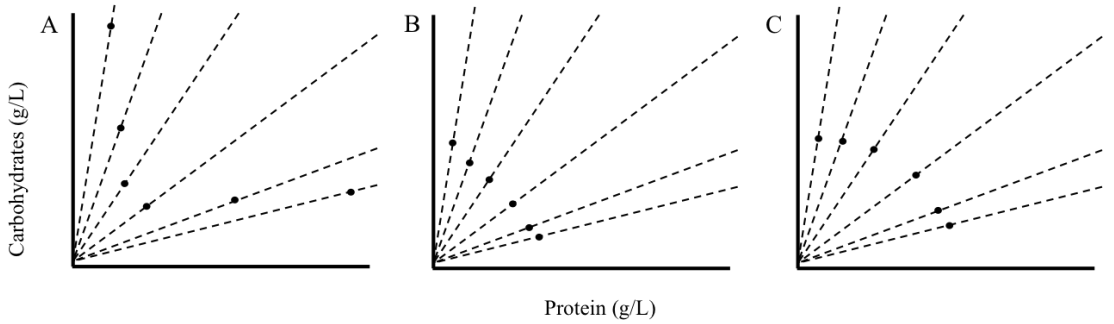

Figure 1.2 – Rules of Compromise. Three examples of rules of compromise, when animals try to reach the intake target for both nutrients, and consequently ingest the other nutrient in excess (A); when animals regulate to ingest a total of booth nutrients that is equal to the intake target (B) and when they ingest till they reach the closest geometric point to the intake target (C). Adapted from Simpson and Raubenheimer, 2012.

Intake target is described as the nutritional point that gives the best nutritional conditions for animals. However, we do not understand completely what are the physiologic consequences for animals due to the over or under consumptions of nutrients.

All types of animals have been shown to actively regulate their nutrient intake, not just in terms of calories but to balance the proportion and quantities of macronutrients, vitamins and salts (Trumper and Simpson, 1993). For humans, body weight is correlated with many aspects of health. Simpson and co-workers, in 2003, showed that humans balance their nutrient intake by prioritizing the acquisition of protein over that of carbohydrate. When faced with a high protein – low carbohydrates diet they over eat proteins in a smaller percentage then they under eat carbohydrates, whereas in a low protein – high carbohydrates diet they over eat carbohydrates to be able to maintain the optimal levels of protein (Simpson *et al.*, 2003). This means that when offered food

high in carbohydrates but low in protein, humans will over-consume carbohydrates to reach their protein target. Further, diets high in protein result in weight loss, since humans will not over-consume protein to reach their carbohydrate target and thus ingest fewer calories overall (Simpson and Raubenheimer, 2005). In mice were also found that when faced with low protein food, their carbohydrates intake also increases (Huang *et al.*, 2013).

The ability to regulate macronutrient intake also extends to invertebrates. Many species of arthropods, like beetles, fruit flies and spiders, regulate their nutrient intake (Mayntz *et al.*, 2005) although they do this using a variety of strategies. The ground beetle (*Agonum dorsale*) selects their prey with respect to its nutritional composition, whereas wolf spiders (*Pardosa prativaga*) adjust the intake of a single prey depending on their own requirements. The web-building spider *Stegodyphus lineatus* extracts from the prey only the required nutrients. Not only do they regulate their nutrient intake, they evaluate which prey would be more suitable to satisfy their nutritional requirements at the time.

Taken together, these studies highlight the power of the nutritional geometry framework in understanding the foraging strategies animals use while foraging. Although exploring intake targets and rules of compromise provide important information regarding how animals make foraging decisions, they do not tell us why they make these decisions. This requires a different approach, using nutritional geometry to explore the effects on developmental and life history related traits.

1.2 Changing Targets in Development and Evolution

Nutritional intake targets change over developmental and evolutionary time (Simpson and Raubenheimer, 1993). In the Mediterranean fruit fly, *Ceratitis capitata*, 2-day old larvae show preference for protein, important for development, whereas at 6 days they tend to prefer carbohydrates, an energy source for the wandering stage (Zucoloto, 1987). Adults also change their intake target; after five days of feeding, female *Locusta migratoria* decrease the amount of protein ingested relative to carbohydrates (Raubenheimer and Simpson, 1999). This change corresponds to the time when tissue growth declines. After mating, the physiological conditions of females change and, coupled to that, the nutritional requirements also change. In *D. melanogaster* mated females show preference for yeast after three days of yeast deprivation. Virgin females kept under the same conditions prefer sugar to yeast (Ribeiro and Dickson, 2010). After mating, females increase egg production. Their preference for yeast is related to the fact that yeast is an important protein source to sustain egg development (Drummond-Barbosa and Spradling, 2001). Thus, alterations in developmental or metabolic programs throughout the animal's life shifts the combination of macronutrients required.

In addition to stage-specific nutrient requirements, species that occupy different trophic levels differ in the particular balance of macronutrients they require. As the trophic levels ascend, the range of macronutrient concentrations available become more

narrow. Further, intake targets increase in protein concentration with increased trophic level. Herbivores (trophic level 2), due to their wider range of macronutrient compositions in their diet tend to prioritize protein acquisition more than predators (trophic level 3). On the other hand, the protein-rich diet of predators cause them to prioritize lipid consumption over protein consumption. Thus, intake target and the foraging strategies used to attain these targets are partly determined by the trophic level an animal occupies.

Within their trophic levels, animals can be grouped into one of two different foraging strategies. Some are able to use a wide variety of substrates (generalists), whereas others are specialists that use a limited range of substrates. Generalist and specialist species differ not only in their breadth of substrates used, but also tend to differ in their tolerance to macronutrient concentrations (Figure 1.3). Due to their greater resource availability, which increases the quantity of available food, generalists tend to achieve the correct balance of nutrients by complementing their diets with a variety of substrates (Bernays and Minkenberg, 1997). Further, when restricted to an unbalanced food generalist species can tolerate ingesting larger quantities of the nutrient in excess to compensate for the scarce nutrient. Specialist species are more prone to nutrient toxicity (Raubenheimer and Jones, 2006).

This difference in diet range has been demonstrated in the generalist, *Schistocerca gergaria,* and the specialist, *Locusta migratoria*, in *ad libitum* conditions on balanced diets the generalist specie ingests more protein than carbohydrates, contrary to the specialist. When are both species fed on unbalanced diets, they both minimize their nutritional error, however generalist ingests a higher excess of protein compared with the specialist. This suggests that the generalist species, *S. gregaria*, is better suited to tolerate excess protein than the specialist (Raubenheimer and Simpson, 2003). Similarly, comparisons between two closely-related caterpillars, *Spodoptera littoralis*, a generalist species, and *Spodoptera exempta,* a specialist species, showed similar results. The intake target of *S. littoralis* sits at a higher P:C ratio than that of *S. exempta*. When faced with unbalanced diets, the generalist over-consumes more protein than the specialist (Lee *et al*., 2002; Lee *et al.*, 2003).

Even within a species, animals can show plasticity in their foraging strategies. *Schistocerca gergaria* can be gregarious or solitarious depending on population density. These two phases differ in their nutrient regulation. Since the gregarious form is more mobile, it encounters a wider range of food sources. Thus, it presents a nutrient regulation pattern similar to generalist species, being able to over eat protein in the presence of unbalanced diet. The solitarious morph consumes less excess protein than the gregarious morph (Simpson *et al.*, 2002).

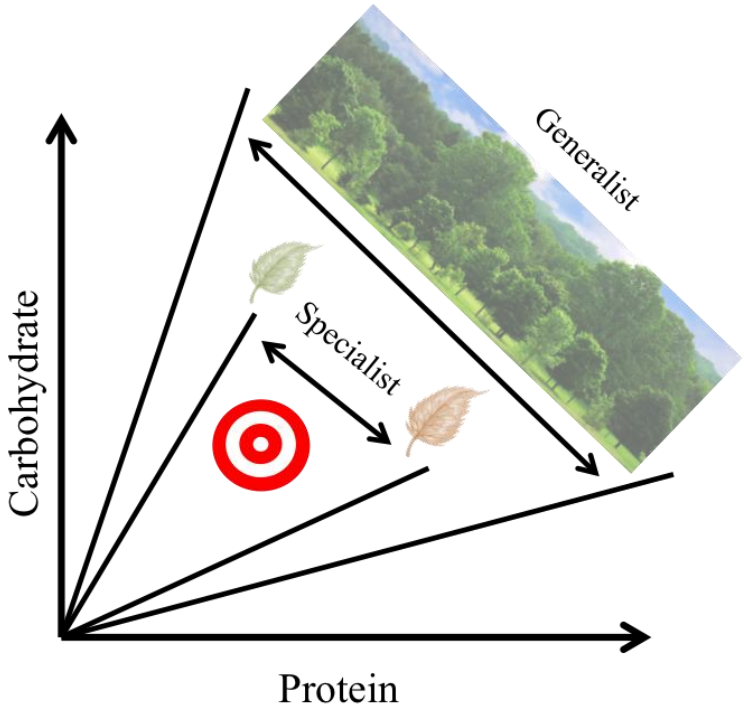

Figure 1.3 – Generalists versus Specialists. Example of when the intake target (red circular shape) is the same, but the means to reach it differs between species. A generalist will use a more broad nutritional landscape to reach the same optimal nutritional levels (intake target) as a specialist, who uses a more specific nutritional landscape. Figure redrawn from Raubenheimer and Simpson, 1999.

The genus *Drosophila* includes a wide range of specialist and generalist species. *D. melanogaster*, with its worldwide distribution and association with primarily urban areas, is typically perceived to be a generalist species as it can colonize a wide range of fruit, fungal and plant material. *Drosophila erecta* is a conditional specialist, prefering to feed on *Pandanus* fruit when it is in season. Other species feed exclusively on a limited range of substrates, like *Drosophila mojavensis*. This species feeds on four species of cacti that grow in the Southern United States and Mexico, and different *D. mojavensis* subpopulations specialize on each of the four cacti (Markow and O'Grady, 2005). Finally, other species are more general in the type of fruits they colonize, but specialize in the time at which they lay their eggs in the fruit. Both *Zaprionus indianus and Drosophila suzukii* prefer to oviposit in ripe, not rotting, fruit (Lachaise *et al.*, 1982 and Nunney, 1990).

Each type of substrate, be it rotting fruit or wounded trees, is colonized by its own particular community of yeast species. In fact, *Drosophila* larvae feed primarily on the yeast communities that colonize their substrate of preference. Yeasts are known to produce species-specific volatile compounds that attract adult flies. In 2014, Schiabor and co-workers found that *D. melanogaster* adults showed different levels of attraction between two strains of yeast (*Saccharomyces cerevisiae*), which had different metabolic products. This suggests that this adult flies were attracted by the yeasts' by-products.

In each of these cases, the substrates *Drosophila* use, have different macronutrient compositions, mainly because of the nature of the substrates but also because of the microorganisms that colonize them. Because different *Drosophila* species colonise different substrates, we expect their nutritional requirements to be adapted to their substrate of preference. Species that feed on ripe fruit may need or tolerate higher levels of simple sugars, since ripe fruit is richer in sugars. These differences in nutritional requirements would certainly be reflected in the response of their development and life history traits to the macronutrient composition of their diet, but also in their foraging strategies.

1.3 Nutrition and its Impacts on Development and Life History Traits

The effects of nutrition on life history traits has been widely studied, especially in the context of longevity and life span (Anagnostou *et al.*, 2010; Baldal *et al.*, 2005; Burger *et al.*, 2010; Fanson and Taylor, 2012; Ja *et al.*, 2009; Lee *et al.*, 2008; Matzkin *et al.*, 2011; Min *et al.*, 2007; Partridge *et al.*, 2005; and others). Many of these studies have explored the role of caloric restriction in longevity, fecundity, body size and other trait. However the quality of food ingested also affects other traits and differs from species to species.

The nutritional geometry framework can be used both to assess how animals balance their macronutrient intake, but also assess how life history traits respond across a broad nutrient space (Fanson *et al.*, 2009). This method implies providing animals with an array of single-choice diets varying in their macronutrient and caloric content. This allows us to map the response of our traits of choice across nutrient space. In Figure 1.4, we present four hypothetical examples of how unbalanced diets composed by a range of protein and carbohydrate concentrations could affect life history traits, such as body size or fecundity. In this example, we use twenty-four different diets that vary in their protein, carbohydrate and caloric contents. The twenty-four diets are represented by black dots. The diets can be grouped as being calorically equivalent (where the sum of carbohydrate and protein is equivalent between diets), or of containing the same (P:C) ratio of protein (dashed lines). In the first example (Figure 1.4-A), the trait responds only to carbohydrates (Figure 1.4-A), where isoclines of the response surfaces increase or decrease with carbohydrate concentration. In the second example, the trait changes with the caloric content of the food (Figure 1.4-B). In the third scenario, the trait correlates only with concentration of protein (Figures 1.4-C), and thus the isoclines follow protein amount and in the final example the trait changes with the P:C ratio (Figure 1.4-D). Of course, the response of any trait can correlate with all four components, protein concentration, carbohydrate concentration, caloric content and P:C ratio.

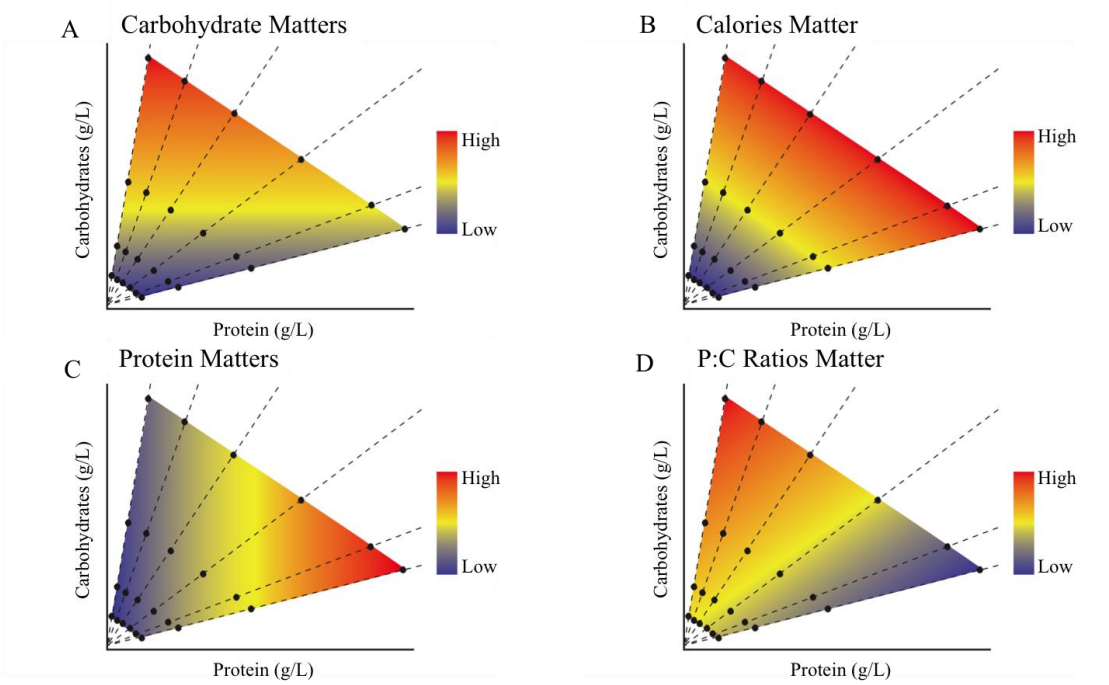

FIGURE 1.4 – Example of how to measure the influence of two nutrients, protein and carbohydrates on life history traits using nutritional geometry framework. In all plots each black dot represents a food type, colours represent the response of the life history trait to the diet and diagonal dashed lines represents the different ratios between protein and carbohydrate (P:C ratios). **(A) When carbohydrates are the principal condition that influences the response**, the colour gradient varies vertically. **(B) Here we show plots that would illustrate the effects on life history traits if they were regulated by calories.** In this case, the colour gradient varies diagonally with food types of the same caloric values. **(C) When proteins are affecting life history trait response** the colour gradient varies horizontally. **(D) When the P:C ratio regulates life history trait response t**he colour gradient varies diagonally with ratio lines.

Nutritional geometry has proven to be a valuable tool for understanding how nutrition regulates life history traits. In 2008, Lee and co-workers applied this framework to a laboratory strain of *D. melanogaster* to explore the effects of protein and carbohydrate composition on longevity, rate of egg production and lifetime egg production. They found that a P:C ratio of 1:4 maximized lifetime reproductive output. On the other hand, longevity was maximized at 1:32 and egg production rate at 1:2. They next offered the flies complementary diets to assess their intake targets and found that flies regulate their intake of protein and carbohydrate toward this 1:4 ratio. Thus, *D. melanogaster* females make foraging decisions to optimize lifetime reproductive success. Similarly, the queensland fruit fly (*Bactrocera tryoni*) shows maximum lifetime egg production at 1:4 P:C ratio, maximum lifespan at 1:32 and maximum egg production rate at 1:1 (Fanson and Taylor, 2012). These females also regulate their intake towards the 1:4 ratio.

The nutritional decisions an animal makes effects not only their fate, but also the fate of their offspring fate. Jaenike, in 1978, concluded that *D. melanogaster* females maximize offspring survival by choosing oviposition sites where larvae will perform

best, termed the oviposition preference – offspring performance hypothesis. Since larvae are not very mobile in the first larval stage, females need to choose oviposition sites considering that the substrate might change with time (Dweck *et al.*, 2013). In fact, females have an innate attraction to lay their eggs in sugary substrates, however they only choose high sugar for oviposition when there is a protein concentration gradient strong enough to first instar larvae follow. In the absence of this gradient, they avoid laying their eggs in sugar (Schwartz *et al.*, 2012).

Many important life history traits are determined in the juvenile stages. During the larval stage, adult body and organ size is determined. The body mass gain during larval stage, which depends on nutritional composition of the food (Sang, 1956), determines adult body size, since growth stops at the onset of metamorphosis. Ovariole number, which is positively correlated with female fertility (Boulétreau-Merle *et al.*, 1982; Klepsatel *et al.*, 2013), is a plastic trait that also varies with nutrition (Fitt, 1990; Kambysellis and Heed, 1971; Leather *et al.*, 1988) and is determined during larval stage (Kerkis, 1931; King, 1970). The many developmental processes that occur during the larval stages are carefully coordinated, and interfering with these processes results in altered developmental time. Because the larval stage is vulnerable to predation, parasitism and competition in a finite food source, larvae are thought to try to minimize their developmental time. Several studies show that larval developmental time is highly affected by food supply (Sokoloft, 1966). By increasing the protein content of the food, developmental time decreases (Anagnostou, 2010).

In our previous studies we found that larvae and adult females of *D. melanogaster* make clear decisions regarding to feeding and oviposition site. Flies showed maximum survival, body size and ovariole number in diets with intermediate protein concentrations and high P:C ratios, whereas developmental time was minimised at an intermediate P:C ratio (1:2). Third instar larvae regulate their intake to minimise developmental time by mixing to different P:C ratios to reach a P:C ratio between 1:4 and 1:2, whereas adult females choose to lay their eggs in a 1:8 P:C ratio and prefer to eat 1:1 P:C ratio (Figure 1.5).

In addition to affecting life-history related traits, nutrition affects a number of developmental processes that result in altered morphology and pigmentation. For instance, nutrition is known to regulate the relative growth of organs to generate differently shaped animals. In dung beetle, *Onthophagus taurus* when a male is well nourished it develops large horns on its head. Poorly-fed males bear disproportionately smaller horns, a phenotype similar to females. This phenotypic difference is determined by nutrition and will also affect males mating strategies; males with large horns guard females and engage in courtship battles whereas males with smaller horns employ a sneaker strategy to mate with females (Emlen, 1994).

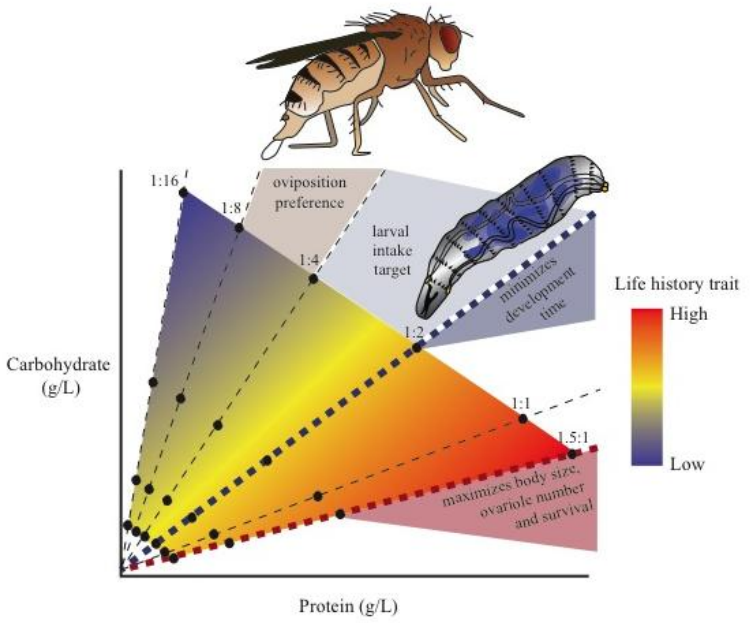

Figure 1.5 – Diagram of how protein and carbohydrates affect life history traits and foraging behaviour of Drosophila melanogaster larvae and adults female (Rodrigues *et al.*, in review).

Similary in the butterfly *Papilio machaon*, pupal colour depends on whether animals pupate on the on cadge, resulting in brown pupae, or on the stalks of the cabbages, generating green pupae (Gardiner, 1974). When *Pieris brassicae* grow in long day conditions produce brown pupae, whereas when they grow in short day conditions the resulting pupae are green. This species also shows variation in pupal colour correlated with diet; when caterpillars feed on green cabbage the pupae are gold in colour but if they feed on white cabbage the pupae are blue/white (Gardiner, 1974). These two species provide good examples of environmentally-induced changes in pupal pigmentation. In the first case, pupal colour appears to be adapted for camouflage. In *Pieris brassicae*, pupal colour variation results from physiological changes in response to environmental cues like photoperiod and nutrition.

Adult cuticle pigmentation in *Drosophila* is widely studied. Although the process is not completely understood, we do know that adult pigmentation changes with temperature and diet. At cold temperatures posterior segments of the abdomen in female are darker than at warmer temperatures (Gibert *et al.*, 2007). Also, during larval stages copper is required in the diet for pigmentation in *D. melanogaster* adults (Zhou *et al.*, 2003). Finally, nutrient sensing through the Insulin/Target of Rapamycin (TOR) pathway has been shown to affect pigmentation of the pupal case in *D. melanogaster* (Shakhmantsir *et al.*, 2014). Because pupal pigmentation is expected to play a role in thermoregulation, desiccation tolerance, mimicry and camouflage (Kronforst *et al.*, 2012), its regulation might bear important impacts on the performance of the animal.

1.4 THE BIOLOGY OF *DROSOPHILA VIRILIS*

The fruit fly, *D. melanogaster*, has been widely used in nutritional studies. With a fully sequenced genome and a wide array of genetic tools, it is a powerful tool for understanding biological processes at the genetic level. Besides being easy to maintain in the laboratory, its life cycle (Figure 1.6) is very quick taking 10 days from egg to adult take at room temperature. In the last years, other *Drosophila* species have been brought into the labs and are being widely used for comparative and evolutionary studies. Here, we focus on how larval nutrition affects life history traits in *D. virilis*. Compared to *D. melanogaster*, *D. virilis* have larger body sizes and are darker in colour. Their life cycle is approximately 12 days at room temperature and is easy to maintain in laboratory conditions.

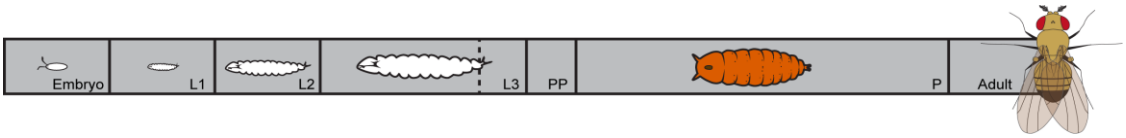

Figure 1.6 – *Drosophila* life stages. L1 corresponds to the first instar larva, after they hatch. They will moult two more times, second instar (L2) and third instar (L3). The last period of the third instar is the wandering phase, where they stop eating. Pre-pupa (PP) is the begging of metamorphosis stage, pupae are still white and eventually they became darker (P). After metamorphosis is complete adult eclose.

Further, *D. virilis* is a specialist, feeding primarily on sap flux in the wild. Virilis group probably originated in warm climates in Asia (Throckmorton, 1977). The ancestral population fragmented into two groups, virilis and montana, as supported by enzyme and chromosomal data (Stone *et al.*, 1960; Throckmorton 1977). The montana phylad occupies cool temperatures regions, whereas the virilis phylad lives in warm to temperate regions (Spieth, 1979). *D. virilis* inhabits the holarctic region. In the wild, it is mainly found in the fluxes of willows and other decaying parts of trees (Throckmorton, 1982). However, sometimes it may be found in fruits in urban regions.

We aimed to understand how macronutrients, including protein and carbohydrate, differentially affect life history traits and developmental processes in generalist versus specialist species. Our previous work uncovered the responses of protein, carbohydrates and caloric content of the larval diet in the generalist species, *D. melanogaster*. Here we compare, in a qualitative manner, our results in *D. melanogaster* to our recent findings in the specialist species *D. virilis*.

2. METHODS

2.1 DROSOPHILA SPECIES

The main focus species of this study is *Drosophila virilis*. This wild-type population came from HHMI's Janelia Farm Research Campus (no stock number assigned). At room temperature life cycle duration is between 12 to 13 days. Like in *Drosophila melanogaster*, males and females body sizes are different and total ovariole number (counting both ovaries) is around 35.

Strains of the following species came from the *Drosophila* Species Stock Center, and include: *Drosophila mojavensis sonorensis* (15081.1352.32), *Drosophila erecta* (14021‐0224.01), *Drosophila pseuoobscura* (14011‐0121.150), *Drosophila tropicalis* 00 (14030‐0801.00) and *Drosophila tropicalis* 01 (14030‐0801.01). *Drosophila willistoni* and *Drosophila nebulosa* strains were provided by Dr. John Jaenike (University of Rochester). All species were adapted to laboratory conditions for several years.

At the Instituto Gulbenkian de Ciência these species are maintained at room temperature on cornmeal molasses food containing 45 g of molasses, 75 g of sucrose, 70 g of cornmeal, 10 g of agar, 1100 ml of water and 25 ml of a 10% Nipagin solution per litre of fly food.

2.2 PERFORMANCE OF EIGHT DROSOPHILA SPECIES

Initially, we planned to compare the response surfaces of life history traits to the macronutrient in the larval diet between several specialist species. We conducted a pilot test using eight *Drosophila* specialist species to see if they would survival on the sucrose/yeast medium used in our nutritional geometry assays.

To accomplish this, we choose a diet which showed high survival in our previous studies of *D. melanogaster*, of 0.72 Kcal/mL and 1:2 a protein to carbohydrate ratio (P:C ratio). Ratios were made up by mixing a 180 g/L solution of Saf-instante dry yeast (Lesaffre, France) and 0.5% agar and a 180 g/L solution of sucrose (Sidul, Santa iria de Azóia, Portugal) and 0.5% agar. Food was autoclaved and we added a 1:50 dilution of propionic acid (Acros organics, Geel, Belgium) and of 10% Nipagen (10% p-hydroxy benzoic acid methyl ester in 95% ethanol, Apex BioResearch Products).

We allowed females of each species to laid eggs for 4-6 hours and 30 eggs were transferred onto a piece of autoclaved paper, which was then placed into vials containing larval diet. We let them develop until adult stage, at constant temperature (25°C) and humidity (60-70%).

Our measurements were, survival from egg to pupa (percentage of larvae that reach pupariation stage) and survival from pupa do adult (percentage of adults that eclose from the animals that pupariated). For these, we counted the number of pupae, every 24 hours, and how many adults emerge from those pupae. We performed 1-10 replicates, depending on the species as for some strains it was difficult to obtain eggs.

2.3 NUTRITIONAL GEOMETRY ASSAY FOR LIFE HISTORY TRAITS IN *DROSOPHILA VIRILIS*

For nutritional geometry assay, we measured the effects of protein, carbohydrate and caloric content of the larval diet on life history traits in *D. virilis* and *D. mojavensis sonorensis*, by changing the quality and quantity of food (see Table 2.1). To accomplish this we made 24 food types, 4 caloric groups (0.18, 0.36, 0.72 and 1.44 Kcal/mL) and for each caloric group 6 P:C ratios (1:16, 1:8, 1:4, 1:2, 1:1 and 1.5:1). These larval diets are made from sucrose and dry yeast solutions, as mentioned above. After all larval diets autoclaved, we added propionic acid and nipagin (1ml/L) to prevent bacteria and fungal growth. This assay was repeated in 4 replicates, 2 replicates at a time.

Similar to pilot test, females were left to lay eggs for 4-6 hours. Eggs were transferred, using a piece of autoclaved paper, into a vial containing the larval diet. Because larval density affects developmental time and body size (Anagnostou *et al.*, 2010), we transferred 30 eggs into each vial. We then let larvae develop at constant temperature (25°C) and humidity (60-70%). We measured survival for *Drosophila mojavensis sonorensis* and survival, developmental time, pharate weight and ovariole number for *Drosophila virilis*.

Measurements:

Survival from egg to pupa: number of individuals that initiate metamorphosis (prepupa).

Larval developmental time: the interval of time from egg until pupariation. For this measure we counted the number of pupae from the vials, twice a day, at 9 am and 5 pm, until all had larvae pupate or died.

Adult body size: a simple proxy for adult body size is to weigh pharate adult pupae (last day of metamorphose). We weigh each pharate adult individually using a Sartorius SE2 ultramicrobalance. We could not distinguish males from females throught the pupal case, and so the two sexes are grouped together.

Adults were transferred together into fresh food vials after eclosion from the pupal case, because this condition stimulates female egg production.

Female Fecundity: ovariole number is a good proxy for female fecundity, as it is correlated with the number of eggs that a female will lay in her lifetime. After 5 days in fresh food with males, females start to produce eggs. In the day of highest egg production, the 6^{th} day after eclosion, we dissected females and counted the number of ovarioles in each ovary.

Table 2.1: Protein and carbohydrate amounts for all 24 larval diets used in this study.

Calories (Kcal/mL)	Ratio	Protein amount per 100 mL food	Carbohydrates amount per 100 mL food
0.18	1:16	0,26	4,15
	1:8	0,48	3,86
	1:4	0,84	3,37
	1:2	1,35	2,7
	1:1	1,9	1,9
	1,5:1	2,25	1,49
0.36	1:16	0,52	8,3
	1:8	0,96	7,71
	1:4	1,68	6,74
	1:2	2,69	5,39
	1:1	3,8	3,8
	1,5:1	4,5	2,97
0.72	1:16	1,04	16,6
	1:8	1,92	15,42
	1:4	3,36	13,48
	1:2	5,38	10,78
	1:1	7,6	7,6
	1,5:1	9	5,94
1.44	1:16	2,08	33,20
	1:8	3,84	30,84
	1:4	6,72	26,96
	1:2	10,76	21,56
	1:1	15,20	15,20
	1,5:1	18	11,8

2.4 NUTRITIONAL GEOMETRY ASSAY FOR PUPAL PIGMENTATION IN *DROSOPHILA VIRILIS*

To quantify the effects of larval diet on pupal pigmentation, we used the same experimental setup as for life history traits outlined above. After adult eclosion, we carefully collected the pupal cases with a wet paintbrush and placed them on a glass slide. They were photographed, ensuring that the white balance was set to the same levels for each image, and then pictures were we analysed in Mathematica using a script developed by Dr. Filipa Alves, from Instituto Gulbenkian de Ciência. This script allows us to draw a transect from the posterior-most part of the operculum to the end of the pupa between the posterior spiracles. The script then measure the RGB values of each pixel on transect (Figure 2.1). We then calculated the average RGB value for each pupa and we measure the distance of those RGB values from white colour, using the Euclidean Distance formula (Equation 2.1).

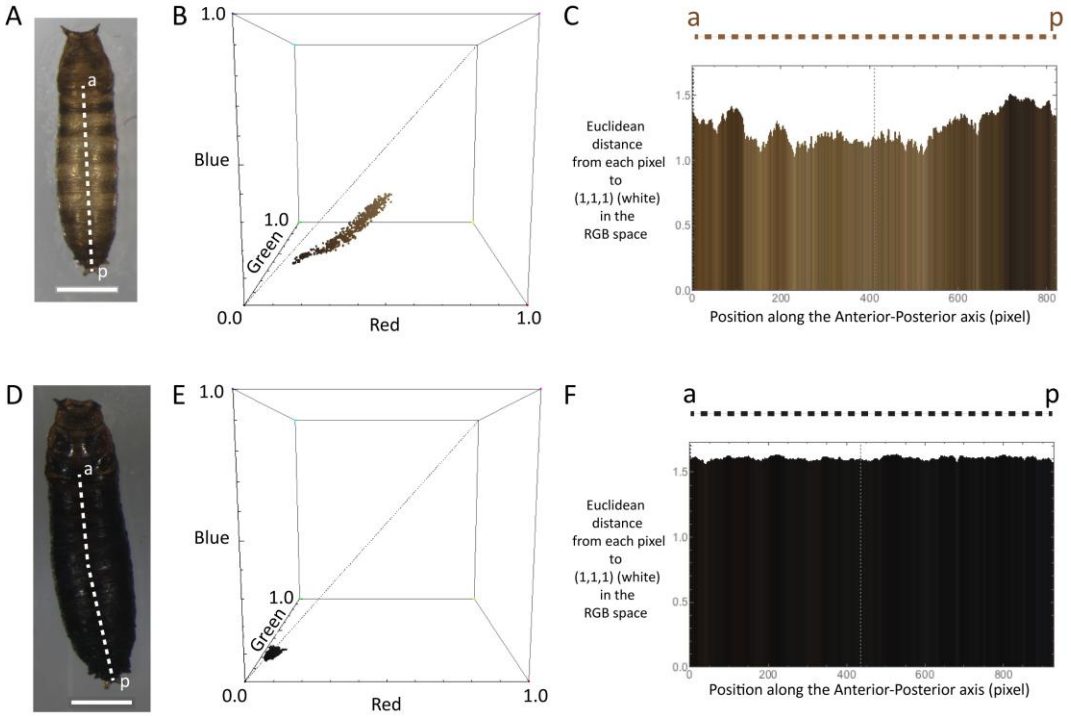

Figure 2.1 – Analyses output from Mathematica. (A) Pupae case from 1:16 P:C ratio (0.72kcal/ml). Dashed line represents the transect use to measure. Scale bar is 0.5mm. (B) Representation of the distance from white of RGB values for each pixel (each dot) throughout the transect line, in a RGB space. (C) Representation of the distance from white of RGB values for each pixel (each line bar) throughout the transect line. (D) Pupae case from 1.5:1 P:C ratio (0.72kcal/ml). Dashed line represents the transect use to measure. Scale bar is 0.5mm. (E) Representation of the distance from white of RGB values for each pixel (each dot) throughout the transect line, in a RGB space. (F) Representation of the distance from white of RGB values for each pixel (each line bar) throughout the transect line.

$$d\,(p,w) = \sqrt{(p1 - w1)^2 + (p2 - w2)^2 + (p3 - w3)^2}$$

Equation 2.1 – Euclidean distance formula used to calculate the distance from white in pupae pigmentation. In this case we are working in a 3 dimensional space. We have three coordinates that codifies a colour for each pupae, *p = (p1, p2, p3)* and the measure how far these colour are from white, which have the coordinates, *w = (w1, w2, w3)*.

2.5 Statistical Analyses for Nutritional Geometry Experiments

All statistical analyses were performed in R (http://cran.r-project.org/) using the nlme, lme4, lmmfit, stats and fields packages and scripts provided by Dr. Nelson Martins (Instituto Gulbenkian de Ciência). All measurements were plotted over a nutrient array defined by protein and carbohydrate concentrations, using thin plate splines.

We estimated the response for developmental time, female and male weight, ovariole number, and distance to white, by fitting a linear mixed effects models, including replictaes as the random effect. For survival, we fit the data with generalized linear model assuming a quasibinomial distribution, to accommodate for the overdispersion of the data, and a logit link function.

2.6 Larval Intake Target in *Drosophila virilis*

Larvae were reared on diet that contained a 1:1 P:C ratio and 0.72 kcal/ml until 3rd instar larvae. Larval diet was prepared in the same manner as for the nutritional geometry assay, but instead of vials we used 60 mm petri dishes. After 4-6 hours egg lay, eggs were transferred to the 1:1 P:C ratios (0.72Kcal/mL) larval diet. Larvae will develop with controlled density (200 eggs per plate), at constant temperature (25°C) and humidity (60-70%).

Within the first 24 h of the L3 stage, we subjected ten larvae to two-choice assays. For this choice assay, we offered larvae the choice between two diets of the same caloric value (0.72 kcal/ml) but differing in P:C ratio. The choices were either between 1:8 and 1:1 or 1:4 and 1.5:1.

To make the assay plate, we fixed 10 lids of 0.5 ml eppendorf tubes into a 60 mm petri dish. Then the plate was filled with a 5% agar solution until the agar solution reached the edge of the lids. We dyed each of the choices either red or blue using 4.5% food colouring (Rayner). The colours will be switched to control for larval colour preference (Figure 2.2).

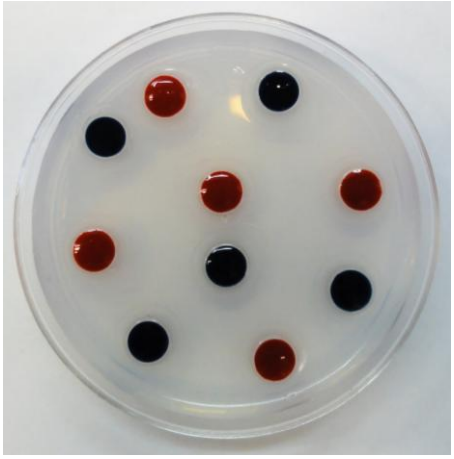

Figure 2.2– Two-way Choice assay plate. Example of an assay plate used for larvae two choice assay. Each color represents one food type, which are inside of 0.5 ml eppendorf lids. Those are fixed with 5% agar solution, filling all the plate.

We left larvae on the assay plate to choose for 1.5, 3 and 6 hours at constant temperature (25°C) and humidity (60-70%). After that, we collected them by flooding the plate with 20% sucrose solution. We then assessed food choice and amount of food, ingested by larvae by spectrophotometer as outlined below.

Food choice: since larvae are transparent and food is coulored, we can distinguish which food each larvae chose by eye. We first counted how many larvae ate each food, or a mixture of both foods (purple color in the gut).

Amount of food ingested: we quantified the amount of food ingested using a spectrophotometer. We processed larvae from each assay immediately after being scored for food choice.

> Spectrophotometer protocol: place larvae into a 1.5 ml tube with 80 µl of ice-cold methanol. Then homogenize larvae and centrifuge at 13 g for 10 minutes at 4°C. Transfer the supernatant to a new 1.5 ml tube and centrifuge at 13 g for 5 minutes at 4°C. Transfer to a 96 well plate 70 µl and measure the absorbance in the spectrophotometer. We used 450 nm absorbance to to quantify red and 600 nm absorbance for blue.

This protocol was repeated at least 20 times for each two-choice assay.

2.7 Adult Female Food and Oviposition Site Preference in *Drosophila virilis*

The experimental setup for this assay was similar to that of the larval intake target assay, except that we used a three-choice design to address female food choice and oviposition site preference. We reared animals from egg to adult in 1:1, 0.72 Kcal/mL diet. Females were left to lay eggs for 4-6 hours, and then 30 eggs were transferred onto autoclaved paper then into the vials with larval diet. After emergence, we transferred all adults to a fresh food vial (the same food where they were reared). When females

started to produce and lay eggs (5-6 days after eclosion), we selected 20 females and 10 males and put them into an assay chamber. The assay plate was similar in design to the larval two-choice assay (Figure 2.3), except instead of flooding the plate with 5% agar we stuck nine 0.5 ml eppendorf lids down into the plate with blu-tack (Bostik). To contain the adults, we taped the plate to a perforated 200 ml plastic cup (assay chamber). During the assay, we offered adults three P:C ratios: 1:1, 1:4 and 1:8.

Figure 2.3 - Three-way Choice assay plate. Example of an assay plate used for female food preference and oviposition site choice, three choice assay. Each color represents one food type, which are inside of 0.5 ml eppendorf lids fixed with blu-tack (Bostik).

Females and males stayed in the cage for 24 hours, at constant temperature (25°C) and humidity (60-70%) and in the dark. Then we froze the adults at -20°C and we assessed female food choice and oviposition site preference. Males were not used, as very few of them ate in the 24 hours tested.

Food choice: we counted how many females chose each P:C ratios, or both foods, by distinguishing the colour of their abdomen.

Oviposition site preference: to assess were females chose to lay their eggs, we counted the number of eggs laid in each P:C ratio.

This experiment was replicated 10 times.

2.8 Statistical Analyses for Food and Oviposition Preference in Larvae and Adult Females

To calculate the preference index for larval intake we used Equation 2.1. We used a Wilcoxon signed rank tests with the null hypothesis of no preference ($\mu=0$) to test for significant preferences. To determine differences between choice, time and colour, we used a pair-wise Wilcoxon rank sum test. Differences between larval intake targets were assessed using a Kruskal Wallis rank sum tests. All this statistical tests were performed in R.

For food and oviposition preference in adult females, we calculated the preference index by the Equation 2.2. We tested for significant preferences using a Wilcoxon signed rank tests with the null hypothesis of no preference (μ=-0.33).

To determine differences between choice, time and colour, we used a pair-wise Wilcoxon rank sum test.

All this statistical tests were performed in R software.

$$Eq.\,2.2.1 - \ Larvae\ PI(food1) = \frac{(\#\ \text{choose food 1} - \#\ \text{choose food 2})}{(\text{total}\ \# - \#\ \text{no choice} - \#\ \text{mix choice})}$$

$$Eq.\,2.2.2 - \ Females\ PI(food1) = \frac{(\#\ \text{choose food 1} - \#\ \text{choose food 2} - \#\ \text{choose food 3})}{(\text{total}\ \# - \#\ \text{no choice} - \#\ \text{mix choice})}$$

Equation 2.2 – Preference index formula. Eq. 2.1 is the formula for larvae preference for food 1, between two-way choice (non choice is 0 value). Eq. 2.2 is the formula for females food preference or oviposition site preference, between three-way choice (non choice is -0.33).

3. RESULTS

3.1 PERFORMANCE OF *DROSOPHILA* SPECIES IN DRY YEAST FOOD

Initially, we had hoped to compare response surfaces to the macronutrient content of the larval diet between species that use different substrates as breeding sites. To do this, we first conducted a pilot study to identify species that would show reasonable survivorship on the simple sucrose/yeast medium that we use for nutritional geometry studies. We selected eight species of *Drosophila* known to differ in their substrate preference. We reared each of these species at 0.72kcal/ml diet at 1:2 P:C ratio, selected as it conferred high survivorship in *Drosophila melanogaster* (Rodrigues et al., in review). We measured the survival from egg to pupa (percentage of larvae that reach the pupariation stage) and survival of pupae (percentage of adults that eclosed from the animals that pupariated).

Our test showed variation in survivorship between species on the sucrose/yeast medium (Figure 3.1). *Drosophila mojavensis sonorensis* and *Drosophila erecta* larvae had a survival of 42% ± 15.3 and 40% ± 13.7, respectively, and of the larvae that reached pupariation 1.8% ± 3 and 4.2% ± 5.8, respectively, eclosed as adults. For *Drosophila willistoni*, 36% ± 7.7 of the larvae pupariated, whereas 71% ± 7.2 of the pupae eclosed as adults. *Drosophila nebulosa* survival showed the opposite pattern to that of *Drosophila willistoni*, 83% ± 6 of the larvae reach pupariation but only 41% ± 11.6 of the pupae reach the adult stage. Finally, the two species with the highest survival were *Drosophila virilis* and *Drosophila tropicalis* 01, a high percentage of larvae reach pupariation stage (96% ± 1.9 and 85% ± 7 respectively) and also a high percentage of the pupae reach to adult stage (99% ± 1.9 and 73 % ± 10.9 respectively).

3.2 NUTRITIONAL GEOMETRY OF SURVIVAL IN *DROSOPHILA MOJAVENSIS SONORENSIS*

Although many species showed low survival in the pilot study, we reasoned that this might be because either the food was too concentrated or the P:C ratio was too high. To test whether changing the protein or carbohydrate content of the food altered survival, we reared a cactofilic species, *Drosophila mojavensis sonorensis*, on the full panel of diets, which include 24 different media differing in protein, carbohydrate and caloric content.

Survival, measured from egg to pupa, was low across all larval diets tested. No significance was found neither to protein, carbohydrates, quadratic components nor protein and carbohydrates product (Table 3.1). Although we can a slightly survival proportion, even if not significantive affected, in the 0.36 kcal/ml diet, high P:C ratios (Figure 3.2).

Since only a small percentage of larvae reached the pupariation stage and of those, more than half did not eclose, we could not measure more life history traits for *Drosophila mojavensis sonorensis*.

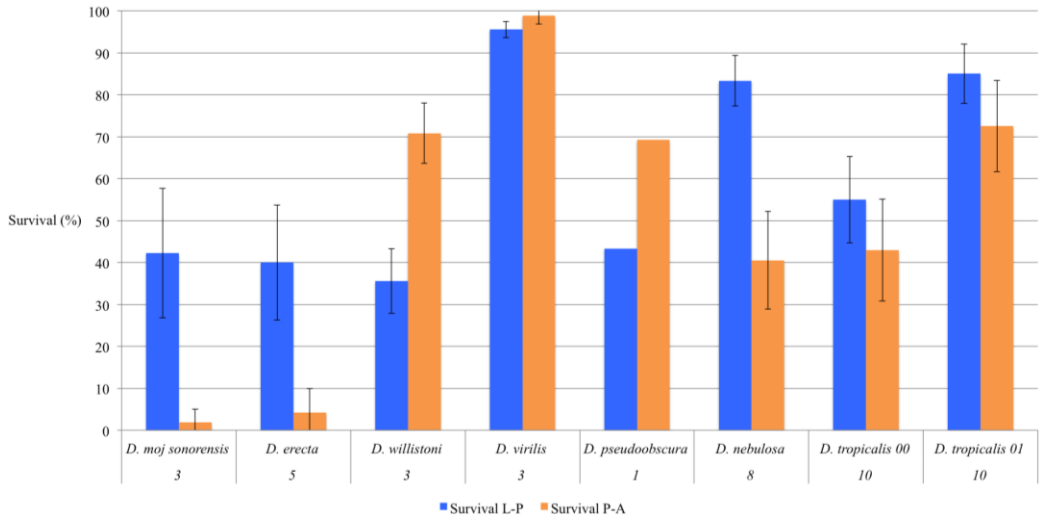

Figure 3.1 – Survival of eight *Drosophila* species in food with sucrose/yeast medium. Eight *Drosophila* species were tested in food with a concentration of 0.72 kcal/ml and a protein to carbohydrate (P:C) ratio of 1:2. Blue bars indicate the percentage of eggs that reached pupariation (Survival L-P), orange bars represent the percentage of pupae that eclosed to adult (Survival P-A). Error bars represent standard deviation of the means. The names of the tested species are in the x-axis and the number below each name indicates the number of replicates per species/strain.

Table 3.1 - Effects of carbohydrate and protein on survival from egg to prepupae in *Drosophila mojavensis sonorensis*. The data was analysed in R by a generalized linear mixed-effects model, assuming a quasibinomial distribution of survival probabilities and a logit link. Significant coefficients are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

Life History Trait		C	P	C^2	P^2	C x P
Survival	β	-0.007	0.930	-0.006	-0.092	-0.012
	t value	0.000	0.032	-0.007	-0.031	-0.004

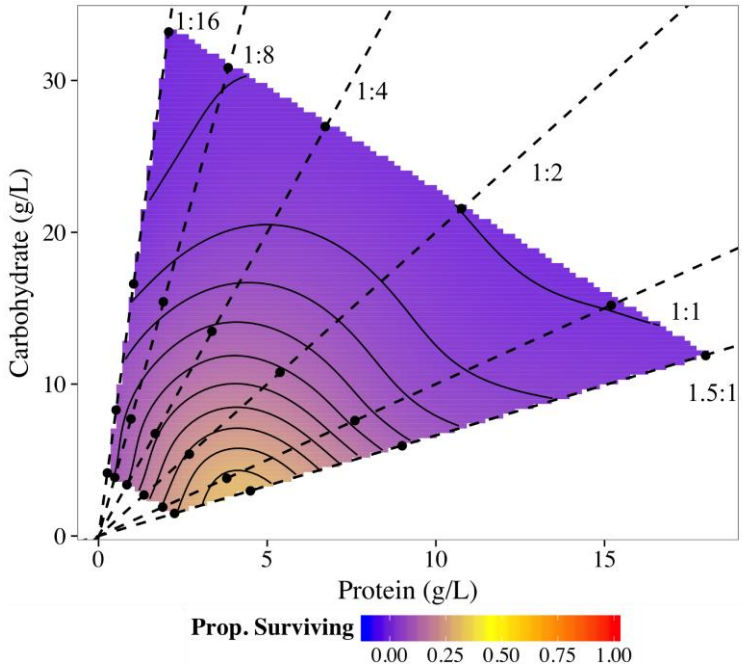

Figure 3.2 – Effects of protein, carbohydrate and caloric content of the larval diet on survival in *Drosophila mojavensis sonorensis*. The coloured area in the plot represents the proportion of animals surviving from eggs to prepupae for the different food types. The proportion varies from blue (the lowest, 0) to red (the highest, 1). Each black dot corresponds to one of 24 larval diets, dashed lines represent protein to carbohydrate ratios (1.5:1, 1:1, 1:2, 1:4, 1:8 and 1:16) and the black lines are isoclines of the response surface.

As showed above *Drosophila virilis* had the best survival, in both life stages, larvae and adults, to unbalance diet on the pilot test. However, this is not the only reason to choose it. This specie is a sap flux specialist, which is very different than the usual rotting fruits. Which makes this a interesting species to look at.

3.3 Nutritional Geometry of *Drosophila virilis*

3.3.1 Survival

Survival from egg to pupae correlated positively with the linear components of protein and carbohydrate, and negatively with quadratic components of protein and carbohydrate and with the cross product of carbohydrate and protein (Table 3.2). For all the unbalance diets survival was maximum at high P:C ratios (Figure 3.3-A). When we examined the shape of the isoclines in the first three caloric concentrations, we observed that the proportion of animals surviving increased with increasing P:C ratios and increasing protein. However, in the 1.44kcal/ml food carbohydrate concentration appeared to be the principle determinant of survival. This resulted in maximum survival proportions at intermediate to high protein concentrations with survival decreasing as protein decreased and as carbohydrate increased from this maximum.

Since the survival for three out of six P:C ratios in 1.44 kcal/ml food was almost zero, we excluded this caloric concentration from the remaining analyses of developmental time, body size and ovariole number.

3.3.2 DEVELOPMENTAL TIME

The faster larvae develop to entre in metamorphosis, the faster they leave the vulnerable stage, larval stage. This is important due to the fact that larvae are expose to predation and resource changes (Krijger *et al.*, 2001). We found a significant negative correlation with linear component of protein and positive correlation with quadratic component of protein (Table 3.2). Overall, the minimum developmental time from egg to pupa was obtained in diets high in protein and intermediate to low in carbohydrates (Figures 3.3-B). The model, including the linear and quadratic components of carbohydrates and protein and their cross product, explained 63,4% of developmental time variation.

3.3.3 PHARATE WEIGHT

In insects, adults do not grow. Because all growth occurs during larval stages larval nutrition is an important determinant of final adult size. Here we measured pharate adult weight as proxy for adult body size (Mirth *et al.*, 2005). In most species, males and females differ in body sizes. However, in this study it was not possible to distinguish the sexes as pharate adults, and we analysed male and female weight together.

We found a significant negative correlation with linear components of carbohydrates and a positive correlation with the quadratic components of carbohydrates (Table 3.2). Further, we found a significant positive correlation with the linear component of protein and a negative correlation with the quadratic component of protein. This resulted in maximum pharate adult weight at the highest protein concentration and highest P:C ratio (Figure 3.3-C). The model explained 61% of the variation in pharate weight.

3.3.4 OVARIOLE NUMBER

During larval development, ovaries start to form and develop the structures that will give rise to the ovarioles in adult females. The number of ovarioles in each ovary correlates with the number of eggs a female lays, and is determined at least in part by larval nutrition (Kerkis, 1931; King, 1970).

Total ovariole number (from both ovaries) correlated positively with the linear component of protein, and negatively with its quadratic component (Table 3.2). Thus, ovariole number is maximized at the highest protein concentrations and the highest P:C ratios (Figure 3.3-D). 23% of the variation in ovariole number can be explained by the model.

3.3.5 COMPARISON BETWEEN LIFE HISTORY TRAITS

We compared the shape of the response surfaces between traits by standardizing each trait to a mean of zero and to unit standard deviations. In addition, because

development time is minimized with increasing protein, we inverted the response surface for developmental time to compare to the remaining traits.

The inverse developmental time surface response differed in shape from the response surfaces for both survival and pharate adult weight (Table 3.3). Inverse development time was smallest at the highest carbohydrate concentrations and lowest P:C ratio, whereas proportion surviving and pharate adult weight showed minimums around low to intermediate carbohydrate concentrations in the lowest P:C ratio. Furthermore, inverse development time increased more steeply with the increase in P:C ratios between 1:16 to 1:4, and showed a broader maximum range. In addition, the response surface for ovariole number differed to pharate weight. The diet with the highest carbohydrate concentration and the lowest P:C ratio resulted in females with the lowest ovariole number. For pharate weight, low to intermediate carbohydrate concentrations in the lowest P:C ratio resulted in the smallest animals. Thus, even though all traits showed maximum values at high protein concentrations and high P:C ratios, the shapes of their response surfaces differed in the conditions that resulted in minimum values.

Table 3.2 - Effects of carbohydrate (C) and protein (P) on four life history traits in *Drosophila virilis*. All traits except survival were analysed in R, by a linear mixed-effects model fitted by maximum likelihood. Survival was analysed by a generalized linear mixed-effects model, assuming a quasibinomial distribution of survival probabilities and a logit link. The significant interactions are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

Life History Trait		**C**	**P**	**C^2**	**P^2**	**C x P**	**R^2**
Survival	β	**1.819e-02**	**4.407e-02**	**-1.090e-04**	**-1.154e-04**	-1.206e-04	
	t value	**0.005 ****	**2.37e-06 *****	**0.002 ****	**0.017 ***	0.062	
Developmental Time	β	6.759	**- 74.737**	0.405	**6.743**	- 1.535	0.634
	t value	1.278	**- 9.169 *****	1.597	**10.185 *****	- 2.069	
Pharate Weight	β	**- 0.067**	**0.290**	**0.003**	**- 0.020**	0.003	0.610
	t value	**- 2.637 ***	**7.376 *****	**2.069 ***	**- 6.325 *****	0.969	
Ovariole Number	β	- 0.031	**2.714**	- 0.026	**- 0.225**	0.043	0.231
	t value	- 0.213	**3.037 ****	- 0.551	**- 3.161 ****	0.511	

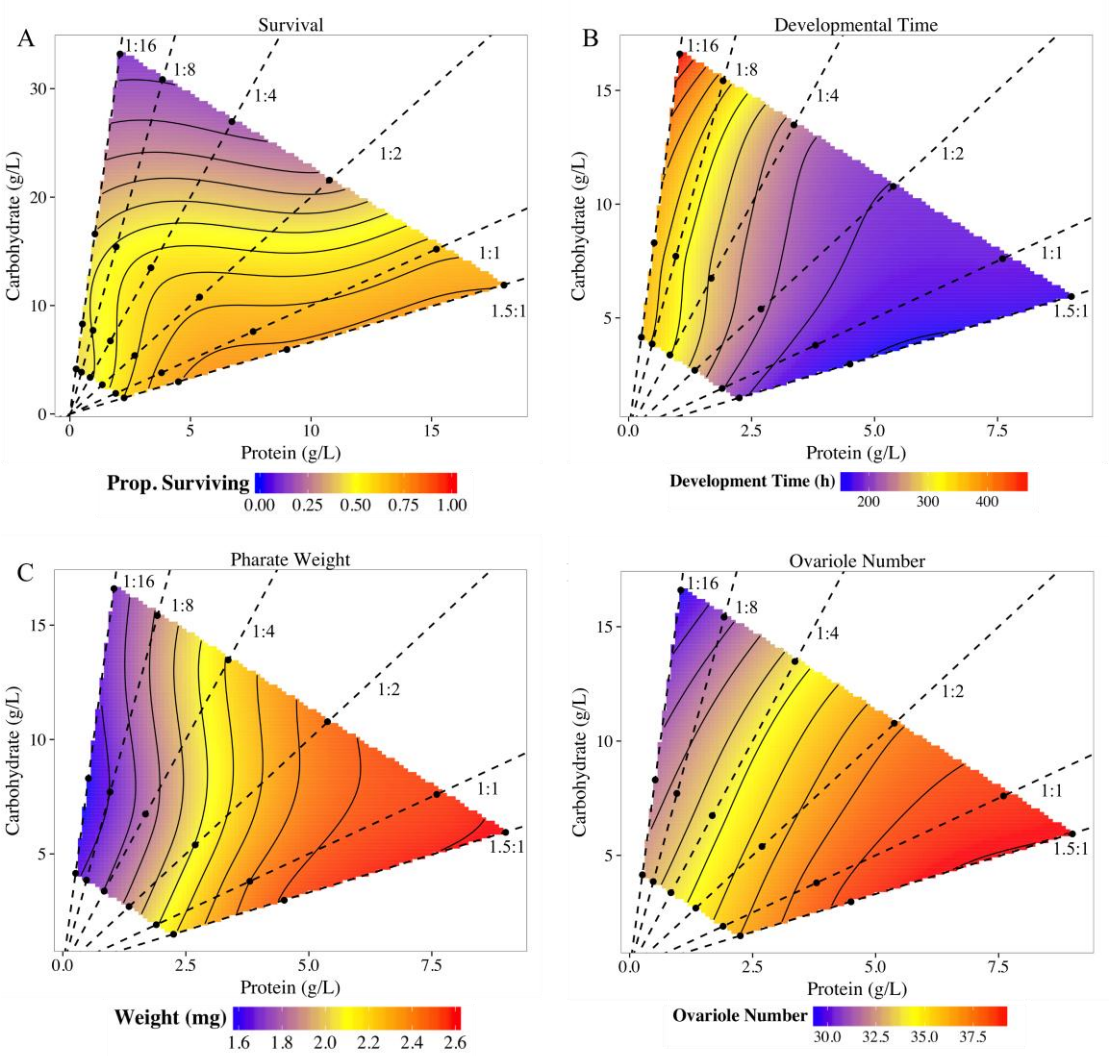

Figure 3.3 - Effects of protein, carbohydrate and caloric content of the larval diet on life history traits in *Drosophila virilis*. In all plots, the coloured area represents the response of a trait to a food type, black dots represent the 24 larval diets, dashed lines represent the protein to carbohydrate ratios (1.5:1, 1:1, 1:2, 1:4, 1:8 and 1:16) and the continuous lines are the isoclines generated using thin plate splines. **(A)** survival from egg to pupa (proportion of individuals that pupariated), for each food type; **(B)** the time that individuals spent to develop from egg to pupariation, in hours, for each food type; **(C)** weight (mg) of individuals at the pharate adult stage, for each food type, and is used as a proxy for adult body size; **(D)** ovariole number is a proxy for female fertility, for each food type.

Table 3.3 – Comparison of the responses to the different food types between life history traits for *Drosophila virilis* (trait A vs. trait B). Data was analysed by partial F tests, comparing the surfaces generated by linear mixed-effects model. The developmental time data was inverted for this comparison. The p-values were adjusted by the Holm method and the significant differences are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

Life History Trait A	Life History Trait B	Degrees of Freedom	L ratio	Adjusted p-value
Survival	Ovariole Number	14	4.020	0.547
Survival	Pharate Weight	14	10.176	0.141
Survival	**Inverse Developmental Time**	**14**	**14.850**	**0.044 ***
Ovariole Number	**Pharate Weight**	**14**	**18.257**	**0.013 ***
Ovariole Number	Inverse Developmental Time	14	12.003	0.104
Pharate Weight	**Inverse Developmental Time**	**14**	**34.365**	**0.000 *****

3.4 NUTRITIONAL GEOMETRY FOR PUPAE PIGMENTATION

While conducting the nutritional geometry experiment, we observed that pupal case showed variation in pigmentation that correlated with the protein content of the food. We found that pupal cases were darker in the diets containing the highest protein concentrations than in conditions with less protein. Also, the pupal cases varied in texture. The darker pupal cases seemed harder and more brittle when compared to the lighter ones, which were softer and easy to damage.

To quantify variation in pigmentation, we collaborated with Dr. Filipa Alves (Instituto Gulbenkian de Ciência). Dr. Alves has developed a Mathematica-based tool to precisely quantify colour from colour-standardized images. We imaged pupal cases from all 24 diets and measured the mean RGB values across a transect from the posterior-most curve of the operculum to the tip of the abdomen for each pupa (Figure 2.1). Then we calculate the distance of coordinates to white coordinates (1,1,1), using Euclidean distance formula (Equation 2.1). Darker colours are further from white, and thus have higher values for distance.

We found that the Euclidean distance from white correlates positively with the linear component of protein and negatively with the linear component of carbohydrate and the quadratic component of protein. (Figure 3.4 and Table 3.4). Thus, pupal cases are darkest at intermediate to high protein concentrations and low carbohydrate concentrations.

Table 3.4 – Effects of carbohydrate (C) and protein (P) on the pigmentation of the pupal case in *Drosophila virilis*. This data was analysed in R using a linear mixed-effects models fit by maximum likelihood. The significant interactions are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

		C	P	C^2	P^2	C x P	R^2
Pupae Pigmentation	β	**- 0.018**	**0.112**	0.001	**- 0.008**	- 0.000	0.827
	t value	**- 2.802****	**11.384*****	1.813	**- 10.558*****	- 0.342	

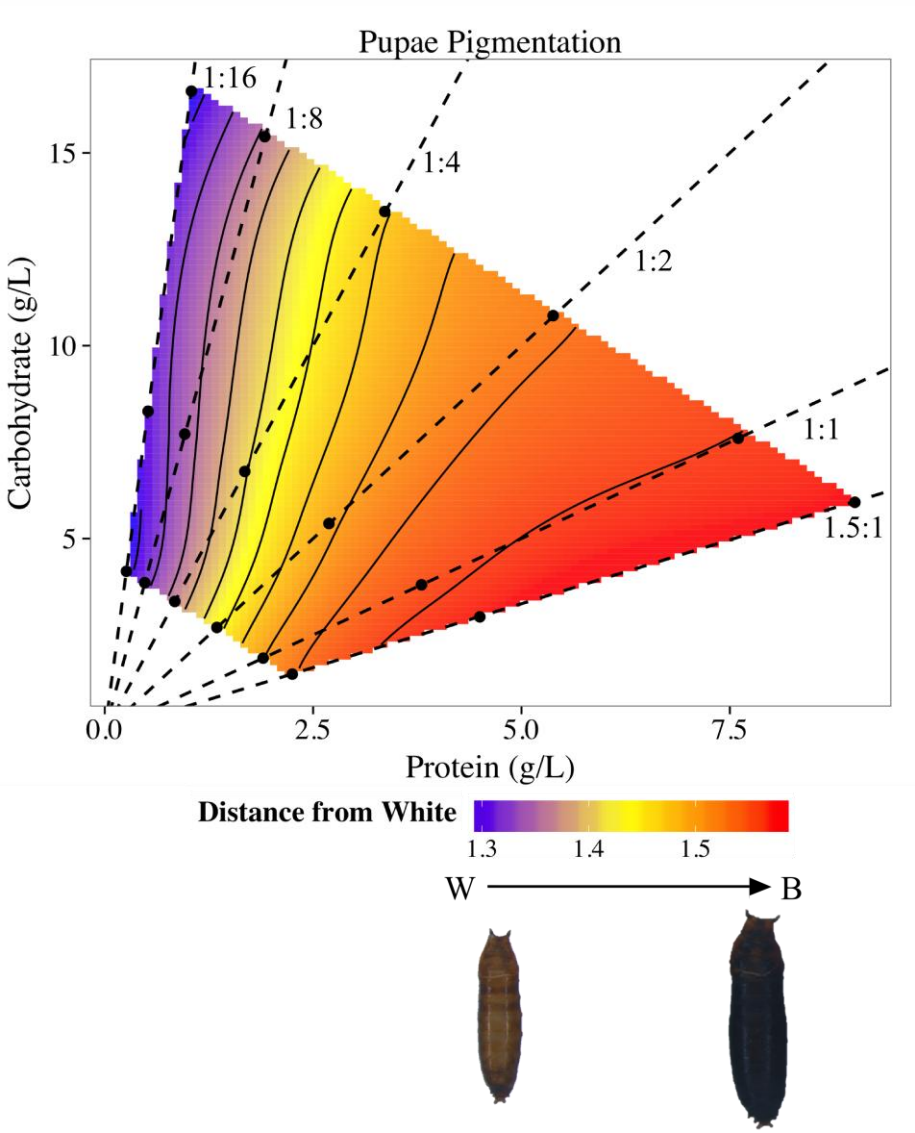

Figure 3.4 – Effects of protein, carbohydrate, and caloric content of the larval diet on the pigmentation of the pupal case in *Drosophila virilis*. The coloured area in the plot represents the distance from white value for each pupae for the different larval diets. Distance from white varies from blue, closest to white (lighter pupae), to red, further from white (darker pupae). Each black dot corresponds to one of the 24 diets. Dashed lines represent the P:C ratios (1.5:1, 1:1, 1:2, 1:4, 1:8 and 1:16) and the continuous lines are the isoclines of the response surface.

3.5 FORAGING BEHAVIOUR

3.5.1 LARVAL CHOICE

To understand how life history traits might drive foraging decisions, we next assessed whether larvae chose food that optimize their development. We did a 2-way choice assay with day one, 3rd instar larvae, offering them a choice between foods of two different P:C ratios (1:1 versus 1:8 and 1.5:1 versus 1:4) but of the same caloric content (0.72 kcal/ml). Because larval food preference could change over time, we analysed larval choice at three times points, 1.5, 3 and 6 hours. Finally, to control for potential colour preference, we alternated the colour of the food in each choice. Although there was no significant colour preference in the 1.5:1 versus 1:4 choice, we observed a significant preference for red in the 1:1 versus 1:8 choice (Figure S1), Wilcoxon signed rank test V=609 and p-value=0.02451).

In both choices, larvae seem to regulate their nutrient intake. Once the range of intake for both nutrients is so narrow, indicates that larvae tightly regulated intake of protein and carbohydrate (Figure 3.5). Thus, resulting in an intake between 1:1 and 1:2 P:C ratios. Especially for the choice 1:1 versus 1:8, larvae regulated carbohydrates intake very carefully. Larvae consumed different amounts of protein, carbohydrates and P:C ratio depending on the choice offered (Table 3.5). Interestingly, larvae offered the choice with the highest protein content (1.5:1 versus 1:4) ingested more protein and more carbohydrate than larvae offered the lower protein combination.

In 1:1 versus 1:8 we found that the amount of protein ingested and the P:C ratio was significantly different over time. However, the amount of carbohydrates ingested did not differ with time (Table 3.6). In the other choice, 1.5:1 versus 1:4 only the amount of protein was significantly different over time (Table 3.6).

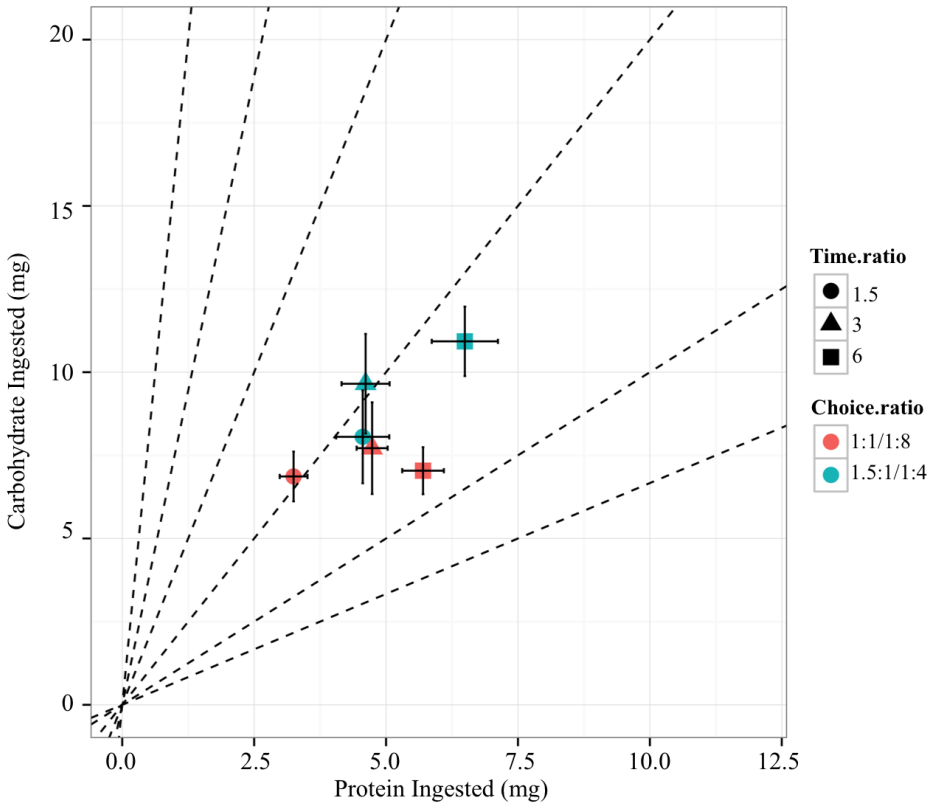

Figure 3.5 – Larval intake target in *Drosophila virilis*. Intake target was quantified using a spectrophotometer. We performed two 2-choice assays. In the first assay, larvae could choose between 1:1 and 1:8 P:C ratios (red symbols). In the second assay larvae could choose between 1.5:1 and 1:4 (blue symbols). We tested the two 2-way choice assays for different time intervals by allowing larvae to choose for 1.5 hour, 3 hours and 6 hours (Time.ratio).

Table 3.5: Comparison of protein to carbohydrate ratio, total protein and total carbohydrate consumed between 2-choice assays (1:1/1:8 and 1.5:1/1:4). Each trait was analysed by a Kruskal-Wallis rank sum test and for all we found significant differences between the choices offered. The significant interactions are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

P:C ratio eaten (mg)
χ^2 =6.6624, df=1, p-value=0.009847 **
Protein eaten (mg)
χ^2 =8.8606, df=1, p-value=0.002914**
Carbohydrate eaten (mg)
χ^2 =19.8828, df=1, p-value=8.234e-06 ***

Table 3.6 – Comparisons of the protein to carbohydrate ratio, total protein and total carbohydrate consumed between the three time intervals (1.5, 3 and 6 hours) for each 2- choice assay (1:1/1:8 or 1.5:1/1:4). For each trait, we did a Kruskal-Wallis rank sum test and a Wilcoxon rank sum pair-wise comparison between each pair of time intervals. The significant interactions are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

Choice offered	Protein eaten (mg)		
1:1/1:8	Time (h)	1.5	3
	3	3.9e-07	
	6	1.3e-10	0.0048
	χ^2 =40.9911, df=2, p-value=1.256e-09 * **		
	Carbohydrates eaten (mg)		
	Time (h)	1.5	3
	3	1	
	6	1	1
	χ^2 =0.071, df=2, p-value=0.9651		
	P:C ratio eaten (mg)		
	Time (h)	1.5	3
	3	0.043	
	6	6.1e-05	0.252
	χ^2 =16.5652, df=2, p-value=0.0002529 * **		
1.5:1/1:4	Protein eaten (mg)		
	Time (h)	1.5	3
	3	0.015	
	6	1.3e-06	1.3e-06
	χ^2 =31.6958, df=2, p-value=1.31e-07 * **		
	Carbohydrates eaten (mg)		
	Time (h)	1.5	3
	3	0.344	
	6	0.052	0.344
	χ^2 =5.7129, df=2, p-value=0.05747		
	P:C ratio eaten (mg)		
	Time (h)	1.5	3
	3	0.52	
	6	0.63	0.46
	χ^2 =2.4186, df=2, p-value=0.2984		

3.5.2 FEMALE CHOICES

So far we found that inverse developmental time, survival, body size and female fecundity are all maximised at the highest P:C ratios. In contrast, L3 larvae regulate the food ingestion to intermediate P:C ratios. This raises the question, do females choose to lay their eggs in P:C ratios that are advantageous for larval development?

In this study, *D. virilis* females were allow to choose, for 24 hours, between three P:C ratios, 1:1, 1:4 and 1:8. We examined the colour of the guts by eye to estimate female food choice and counted the number of eggs in each food to assess oviposition site choice. To account for colour preference, we alternated the colour of each P:C ratio between the three dyes. For food choice, females avoided blue (Figure S2 and Table S1). They showed no preference for the green or red (Figure S2 and Table S2). Females also did not show any preference for colour for oviposition (Figure S2 and Table S2).

Females food choice seemed to be affected by P:C ratio, we found a significant difference between 1:8 and 1:4 (Table 3.7), it seems that females tend to avoid the 1:4 P:C ratio (Figure 3.6-A). However, 68.7% of all females tested did not choose any P:C ratio (Table S2). Females did not show any discernable preference in P:C ratios for oviposition site (Kruskal-Wallis rank sum test $\chi^2 = 0.4027$, df=2, p-value=0.8176(Figure 3.6-B). However we can not assume that female do not take decisions about oviposition site, once the number of eggs lay, on average, per plate was only 49.75 eggs.

Table 3.7 - Female food choice. Kruskal-Wallis rank sum test showed a significant effect of P:C ratio on females food choice (χ^2 =11.092, df=2, p-value=0.003903). The table shows the p-values of the Wilcoxon rank sum pair-wise comparisons between P:C ratios offered. The significant interactions are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

Preference Index for females food choice		
Choice offered	1:1	1:4
1:4	0.46	
1:8	0.13	**2.9e-05 *****

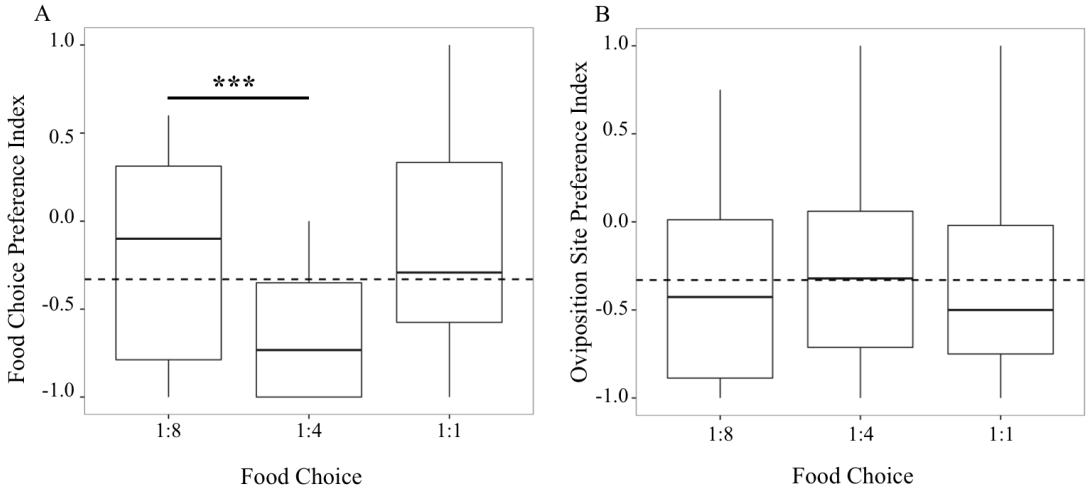

Figure 3.6 – Female food and oviposition site preference when offered 1:8, 1:4 and 1:1 P:C ratios. (A) Female preference index was calculated by (# females that ate food 1- # females that ate food 2 - # females that ate food 3) / (total # females - # females that did not eat - # number of females that ate more than one food). (B) Female oviposition site preference index (preferred food to lay eggs) was calculated by (# eggs in food 1- # eggs in food 2 - # eggs in food 3) / (total # eggs laid). In both plots the dashed line represents the no-choice value (- 0.33). The asterisk indicate the difference between 1:8 and 1:4 choice, Wilcoxon rank sum pair-wise comparisons (2.9e-05 ***) in female food choice

4. DISCUSSION

In this study, we sought to better understand how macronutrient in the diet influence life history traits, developmental processes and foraging decisions in specialist species. To accomplish this, we measured the importance of proteins and carbohydrates on life history traits, pupal pigmentation and foraging behaviour in larvae and adult females of the tree flux feeder, *D. virilis*. For this specialist species all the traits were maximised at high P:C ratio, although larvae do not regulate intake target to maximise any of those traits. With *D. melanogaster* previous data, we can increase our knowledge about differences between generalist and specialist species.

4.1 *DROSOPHILA MOJAVENSIS SONORENSIS*

D. mojavensis sonorensis, is a substrate specialist, which feeds on fermented cactus tissue (Fellows and Heed, 1972). The host cactus of this species is the *Stenocereus thurberi* (Pfeiler, 2009), commonly named the organ pipe cactus.

D. mojavensis sonorensis showed low survival on all diets differing in protein and carbohydrate content and calories. Neither proteins nor carbohydrates played any role on this trait. This suggests that the simple sucrose/yeast medium lacks a critical component necessary for their development and growth.

It is known that cacti contain species-specific allelochemical compositions (Fogleman and Danielson, 2001). *Drosophila* species that use these cacti are thought to be adapted to the allelochemical composition of their host (Etdges and Heed, 1987). For example, *Drosophila pachea* specializes on senita cactus (*Lophocereus schottii*). Unlike most *Drosophila* species *D. pachea* cannot biosynthesize the steroid hormone ecdysone from cholesterol, a hormone important for regulating development and molting. This is because *D. pachae* lacks a key enzyme, Neverland, that converts the cholesterol metabolites to lathosterol. This species is an obligate senita cactus feeder, as the senita cactus provides a source of lathosterol necessary for its development (Lang *et al.*, 2012).

D. mojavensis sonorensis is a subspecies from *D. mojavensis* group. This subspecies, originated due a specialization on different yeast species that colonizes different cacti. Although this species has been in the laboratory for several years, and it is easy to maintain with *Saccharomyces cerevisiae*, the yeast species used in lab food. Since, yeast is the major source of sterols for *Drosophila* (Carvalho *et al.*, 2010), in unlikely that our results are due to a lack of sterols. However, we do know that some component is missing. To over come this problem, we suggest to use unbalance diets with ingredients used in lab food for a nutritional geometry framework to analyse the effects on life history characters. It would also be interesting, with the same method to analyse a variety of yeast species. This way, we might identify a yeast species that confers better survival.

4.2 *DROSOPHILA VIRILIS*

Since *D. virilis* lays its eggs primarily in tree wounds and/or sap flux, we expected that this species would show a different response to the macronutrient composition of its diet than the widely studied generalist species, *D. melanogaster*. *D. virilis* can only colonize sap flux when this is exposed to the outside were it can be colonised by yeast. Since plant flux is relatively nutrient-poor, with cactus flux showing lower nitrogen and phosphorus content than fruit, we would expect *D.* to be more tolerant to low nutrient media (Jaenike and Markow, 2003; Markow *et al.*, 1999).

4.2.1 SURVIVAL

Our data showed that both, the protein and carbohydrate content of the larval diet play an important role in survival in *D. virilis*. Survival was maximum at high P:C ratios, independent of the caloric content of the diet. However diets with high caloric (1.44 Kcal/ml) content and low P:C ratios (1:4, 1:8, and 1:16), survival was low. This suggests that high concentration of carbohydrate in larval diet is lethal for *D. virilis*.

This contrasts with what has been previously described regarding the effect of macronutrients in the larval diet on life history traits *D. melanogaster*. Anagnostou and co-workers (2010) showed that initial yeast cell mass, which is equivalent to food quantity, in the larval diet does not affect *D. melanogaster* survival. Using a different approach, Schwarz and co-workers (2014) measured the effects of adding sucrose to standard food in *D. melanogaster* survival. They found no significant difference between standard food and standard food with sucrose on egg to adult survival. In our previous study with *D. melanogaster*, we found that survival from egg to pupa was maximized at high P:C ratios (1.5:1 and 1:1). As a matter of fact, high content calorie diet do not showed such a drastic effect on survival (Rodrigues *et al.*, in review). As expected, the specialist specie *D. virilis* response to a wide range of unbalance food differs form the generalist *D. melanogaster*, showing that *D. virilis* seems to be more sensitive to high nutrient variations on diets.

4.2.2 DEVELOPMENTAL TIME

We found that developmental time is minimised at high P:C ratios and increases drastically in diets with low P:C and high carbohydrate contents. Larvae are vulnerable to predation and development can be compromised due to changes in food source. For instance, in *D. mojavensis* developmental time increases with the ageing of the necrotic cactus tissue (Etges and Heed, 1987). Thus, faster developmental time is thought to be advantageous in several situations, as in competitive ability (Krijger *et al.*, 2001).

D. melanogaster larvae develop faster in a standard food diet supplied with sucrose that in a standard food diet without sucrose (Schwarz *et al.*, 2014). This outcome was also found in our previous study, whereas *D. melanogaster* larvae minimized they developmental time at 1:2, an intermediate P:C ratio (Rodrigues *et al.*, in review). In the present study, *D. virilis* larvae develop faster at extreme high protein content diet. Thus,

suggesting that high-protein content diet is better for our specialist species, whereas for the generalist, *D. melanogaster*, a more balance P:C ratio is better.

4.2.3 BODY SIZE

Several studies have reported, in *D. melanogaster*, that protein diets are essential for a proper larval development (Mirth and Riddiford, 2007; Mirth and Shingleton, 2012 and Koyama *et al.*, 2013). Increasing protein content in *D. melanogaster* diet would increase adult body size (Mirth and Shingleton, 2012). The importance of body size is because is related to a reproductive success (Lefranc and Bundgaard 2000; Partridge *et al.*, 1987; Partridge and Farquhar 1983).

In our study, we found that body size is maximised at high P:C ratios in *D. virilis*. Actually, body size increases with increasing protein concentration. Carbohydrates also play a role, although of small effect on body size. In *D. melanogaster*, maximum body size was accomplished in high protein diets. However, in other insects, such as *Spodoptera exempta*, dry pupal mass increases by increasing carbohydrates content in the diet (Lee *et al.*, 2003 and Lee *et al.*, 2004). The Lepidoptera, *Spodoptera exempta* and *D. virilis* are both specialist species and yet both species respond differently to macronutrients.

4.2.4 OVARIOLE NUMBER

Ovariole number is positively correlated with the number of eggs that a female will produce (Boulétreau-Merle *et al.*, 1982; Klepsatel *et al.*, 2013) and is determined during larval stage (Kerkis, 1931; King, 1970). For *D. virilis* ovariole number maximised at high P:C ratios, whereas in our previous study for *D. melanogaster* ovariole number is maximised at high P:C ratio (1.5:1) but for intermediate protein content. We believe that protein is playing an important role, which is expected since ovariole formation during larval stage requires cell division, proliferation and differentiation (Godt and Laski, 1995; Sahut-Barnola *et al.*, 1995; Sahut-Barnola *et al.*, 1996). Carbohydrates seem to not affect ovariole number, except for very high content.

In generally, for the traits above, protein seems to be a key nutrient. High content of carbohydrates also affected some traits, such as, survival and body size in a negative way.

In our previous study, we were focus only in an outbred population of *D. melanogaster*. When we compared the response between both species, we found clear differences between them. The most obviously differences are regarding survival and developmental time. In *D. melanogaster* survival was maximum at intermediate protein content, but high P:C ratios, whereas in *D. virilis* we found higher survival at high P:C ratios. Developmental time in *D. melanogaster* was minimized at intermediate P:C ratios, whereas in *D. virilis* developmental time was minimized at high P:C ratios. Body size and ovariole number in both *D. melanogaster* and *D. virilis* were maximized at high P:C ratios (Figure 4.1).

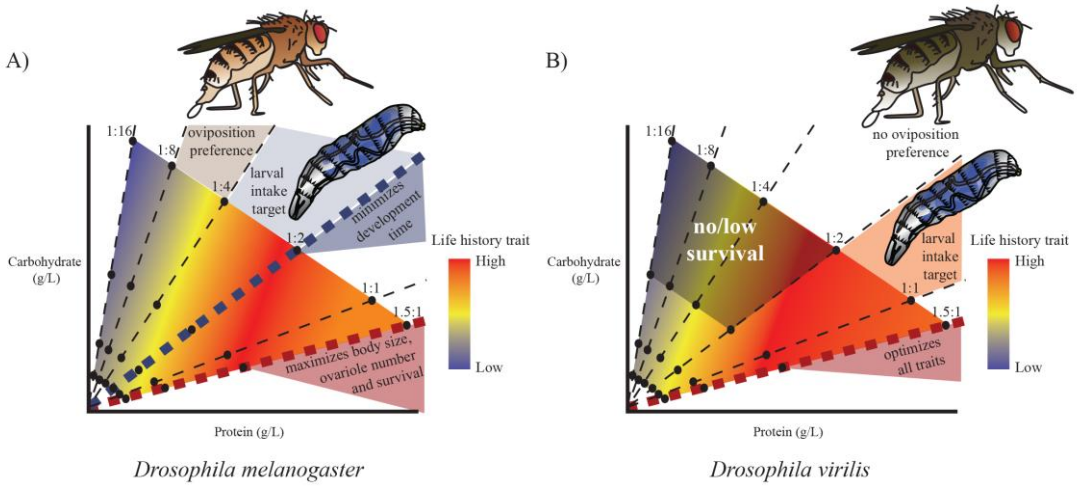

Figure 4.1 – Summary figures for macronutrients effect on life history traits and foraging behaviour. (A) *Drosophila melanogaster*, maximizes body size, ovariole number and survival at 1.5:1 P:C ratio and minimises developmental time ate 1:2 P:C ratio. Larvae regulate the intake target to 1:2/1:4 and females lay eggs on 1:8 P:C ratio (Rodrigues *et al.*, in review). (B) *Drosophila virilis* all traits were optimised at 1.5:1 P:C ratios, whereas larvae regulate intake target to 1:1/1:2 P:C ratio. Females did not show any preference.

4.3 PUPAL PIGMENTATION

During nutritional geometry assay we found that the pupal cuticle showed variation in its pigmentation with increasing protein concentration in the diet. The darkest pupae were found at the highest P:C ratios, whereas the lightest pupae occurred at low P:C ratios. In al larval diets, prepupae are white and then start to became darker. It would be interesting to explore the dynamics of pupal pigmentation to see whether the darker pupae undergo a longer pigmentation phase or if they pigment faster than the lighter pupae. Furthermore, we noted a difference in the hardness of the pupal cases. The darkest pupal cases were more brittle while the lightest cases were deformable. It would be interesting to investigate if variation in pupal pigmentation correlated with differences in the thickness of their pupal case.

In addition to its developmental dynamics, it would be interesting to explore the potential advantages this variation in pigmentation might provide. *D. virilis* is a holarctic species, occurring across a range of temperatures and forest types. Pigmentation is known to play a role in thermoregulation and desiccation tolerance in several species of *Drosophila* (Kronforst *et al.*, 2012). Pigmentation in adult *D. melanogaster* shows temperature plasticity, with flies reared in colder environments being darker than those reared at warmer temperatures (Gibert *et al.*, 2007; Kennell *et al.*, 2012). Potentially, darker pupal cases could be in cold climate and against desiccation, or lighter pupae in warm climates. Although this appears to be a plausible argument for temperature-induced plasticity, this does not explain the nutritional plasticity in pupal pigmentation. We can also consider that this might work as a camouflage. Another interesting possibility is whether this pigmentation differences would, actually, be related with different thickness of pupal case in terms of giving protection against parasitoid wasps. In this case, we speculate that a thicker pupal case

would prevent the wasps pricking, contrarily to a thinner pupal case.

The genetic mechanisms that alter pigmentation in response to nutrition have been partially described in *D. melanogaster*. Insulin/Target of Rapamycin (TOR) pathway is involved in growth, and other processes, and is sensitive to nutrition. In *D. melanogaster*, activation of this pathway represses FOXO resulting in darker pupae and adults flies (Shakhmantsir *et al.*, 2014). Although this link between the insulin/TOR pathway appears compelling, we understand little about how the activity of this pathway might affect pigmentation. The pigment synthesis pathway gives rise to colour (Figure 4.2) (Kronforst *et al.*, 2012), which converts tyrosine to dopa-melanin or dopamine-melanin through the activity of several well-described enzymes. Potentially, the insulin/TOR pathway could regulate pigmentation by regulating the amount of substrate available for conversion, or by regulating the amount and/or activity of the enzymes in the pathway.

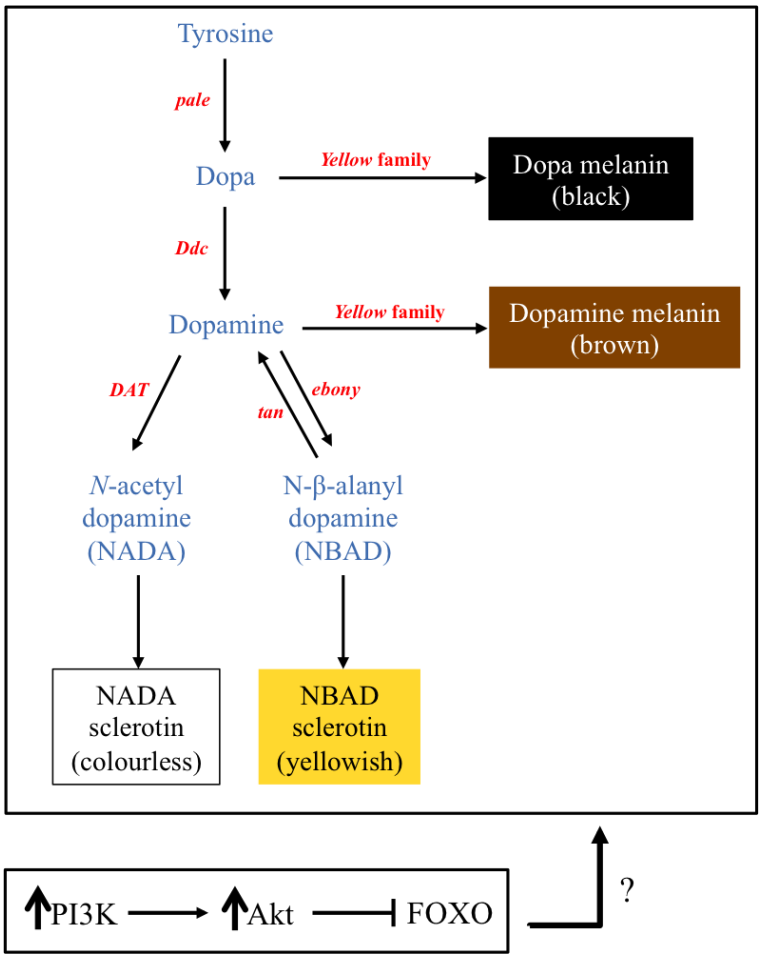

Figure 4.2 – Simplified scheme of the pigment synthesis pathway. In blue are represented intermediate metabolites and in red the enzymes that produce them. When PI3K is overexpressed (from Insulin/Target of Rapamycin (TOR) pathway), Akt increases which is a suppressor of FOXO. Without FOXO, adult and pupal pigmentation increases. Adapted from Kronforst *et al.*, 2012.

4.4 FORAGING BEHAVIOUR

4.4.1 LARVAL CHOICES

When we offered 3rd instar larvae to two unbalanced diets, they regulated their intake towards a specific intake target. Our data showed that these larvae regulated their intake for both protein and carbohydrate (Figure 3.5) within a tight range. This suggests that *D. virilis* larvae avoid eating excessive amount of these macronutrients. In contrast, our results from *D. melanogaster* suggest they tightly regulate their protein intake, but show greater variation in their carbohydrate intake (Figure 4.1-A).

Interestingly, larvae regulate their intake towards a P:C ratio between 1:2 and 1:1. In our nutritional geometry assay, all the traits were maximised at high P:C ratios. The maxima tended to occupy a broad range of P:C ratios, and often overlapped with the 1:2 P:C ratio. The fact that larvae did not target their intake to the highest ratios, 1:1 and 1.5:1, suggests that there could be additional trade-offs of consuming the highest P:C ratios that we did not measure. Alternatively, in foraging choice assays we used 3rd instar larvae, whereas in nutritional geometry experiments we measured the consequences of a particular diet provided throughout all larval stages on life history traits. In previous studies, nutritional requirements have been shown to change with developmental stage. Potentially, in earlier stages larvae opt for higher P:C ratios than in the 3rd instar. Finally, target intake can also change with time, a phenomenon known as a moving target. For both of our choices assays, both the amount of protein eaten and the P:C ratio ingested changed with time. In *Ceratitis capitata,* before wandering stage their intake change to a more carbohydrates consumption (Zucoloto, 1987). Here we found a possible moving intake target, in both choices, mainly for protein. Thus, suggesting that over time their protein consumption may increase. Regarding carbohydrates consumption we did not find any clue for a moving target. Are carbohydrates more important, leading to a very thigh consumption regulation?

An interesting experiment would be to let *D. virilis* larvae feed freely on one single unbalanced diet, for a specific amount of time and then measure how much did they eat. If larvae try to maximise survival or pharate weight traits, then we expect to see a regulation for proteins without regulate carbohydrates consumption, resulting in over or under intake of carbohydrates (Figure 4.3 – A). However, if larvae decide to minimise for developmental time and maximise ovariole number, then we will see a carefully regulation for both nutrients (Figure 4.3 – B). The hypotheses are based on nutritional geometry results, since for survival and pharate weight carbohydrates seem to have a small effect, whereas for developmental time and ovariole number high content of carbohydrates had negative effects.

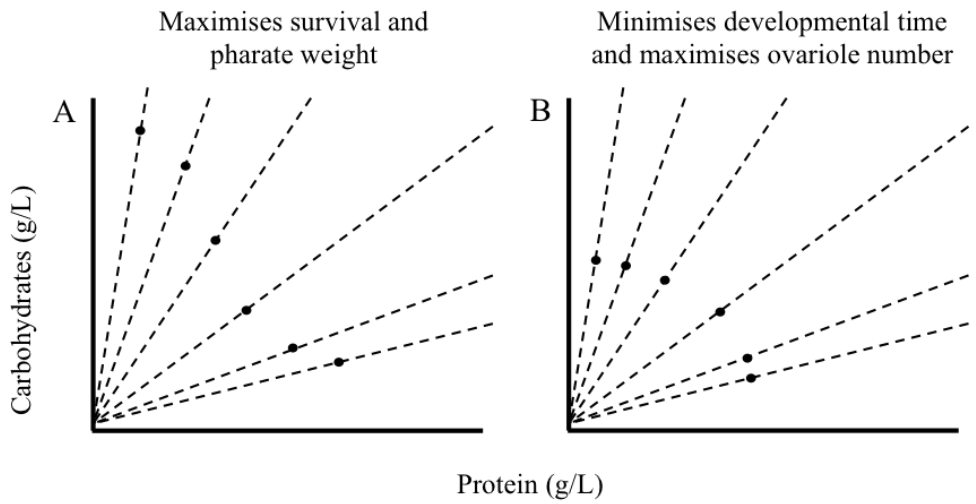

Figure 4.3 – Two hypothetical larvae intake response. For both plots dashed lines represents P:C ratios, whereas black dots represents larvae intake. (A) Representation of when larvae feeding *ad-libitum* throughout a P:C ratio would regulate consumption for only one of the nutrients, protein in this case, maximising survival and pharate weight. (B) Here we hypothesis that larvae would regulate for both nutrients, proteins and carbohydrates, minimising developmental time and maximising ovariole number.

4.4.2 FEMALE CHOICES

Several studies have showed that females decide to lay their eggs depending on the nutritional composition of the substrate and near food sources. In *D. melanogaster*, females showed an obvious preference between P:C ratios for oviposition site choice. When offered three different P:C ratios, 1:1, 1:4 and 1:8 (0.72 kcal/ml), females laid a greater proportion of their eggs in 1:8 food and ate almost exclusively the 1:1 diet. This seems to go against the Jaenike hypothesis, oviposition preference – offspring performance, whereas females will choose to lay their egg in a substrate that would give the best conditions for larval develop (Jaenike, 1978), however since substrates are nutritionally dynamic females may predict those changes throughout larvae development.

When we offered *D. virilis* females a choice between three P:C ratios, 1:1, 1:4 and 1:8 at 0.72Kcal/mL, they showed a no preference for food choice and oviposition site. In terms of food choice, this could be because very few females ate during the duration of our assay, with 68.7% of them not eating any diet. Also, females showed no preference between P:C ratios in terms of oviposition. This may indicate that in *D. virilis,* females do not use the P:C ratio of the substrate to make oviposition choices, but rather rely on other cues.

Regarding feeding choice, we believe in two statements, or this result is due to a deficiency in experimental set or *D. virilis* females do not make any decision.

In our experimental set, we used 0.5 mL eppendorf lids in the assay plate (Figure 4.3 - A), which has small amounts of food. Within the 24 hours of assay there is a big probability of dehydration of food, which may affect the quality of food and consequently females decision. An alternative to that is to use a whole food plate like the drawn from Figure 4.3 - B.

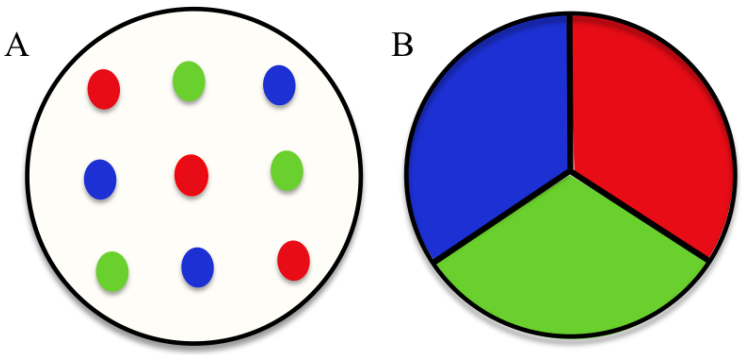

Figure 4.3 – Assay plates for female's food choice and oviposition site preference. (A) This is the assay plate used in our study. (B) This is the assay plate suggest to improve our experimental set.

CONCLUSION

In our study we found that protein and carbohydrate concentration in the larval diet plays an important role in regulating life history traits of *D. virilis*, with all traits showing optimal values at the highest protein concentrations. Furthermore, we find that although all traits were similar in their optimal values, we could distinguish two types of responses depending on conditions that generated the worst performance. Where survival and pharate adult size were worst in the low P:C, low-intermediate carbohydrate diets, development time was longest and ovariole number was lowest at low P:C and high carbohydrate concentrations. In comparison to our previous data from *D. melanogaster*, we find qualitative differences in the way life history traits respond differently to the protein and carbohydrate content in the larval diet in *D. virilis*. In addition, we found that the pigmentation of the pupal cuticle varied with protein content on the diet, becoming darker with increasing protein. We propose that this might results of interactions between the insulin/TOR pathway and the melanin pathway. Finally, we find that larvae tightly regulate their protein and carbohydrate ingestion, whereas females did show any type of preference between different diets. Our finding indicates that depending on species feeding strategies, whether they are generalist or specialist species nutritional requirements and foraging strategies will differ in unbalance nutritional environments.

BIBLIOGRAPHY

Anagnostou, C., Dorsch, M., & Rohlfs, M. (2010). Influence of dietary yeasts on Drosophila melanogaster life-history traits. *Entomologia Experimentalis et Applicata*, *136*(1), 1–11. doi:10.1111/j.1570-7458.2010.00997

Baldal, E. a, van der Linde, K., van Alphen, J. J. M., Brakefield, P. M., & Zwaan, B. J. (2005). The effects of larval density on adult life-history traits in three species of Drosophila. *Mechanisms of Ageing and Development*, *126*(3), 407–16. doi:10.1016/j.mad.2004.09.035

Bernays, E. A., & Minkenberg, O. P. J. M. (1997). INSECT HERBIVORES: DIFFERENT REASONS FOR BEING A GENERALIST. *Ecology*, *78*(4), 1157–1169. doi:10.1890/0012-9658

Boulétreau-Merle, J., Allemand, R., Cohet, Y., & David, J. R. (1982). Reproductive strategy in Drosophila melanogaster: Significance of a genetic divergence between temperate and tropical populations. *Oecologia*, *53*(3), 323–329. doi:10.1007/BF00389008

Burger, J. M. S., Buechel, S. D., & Kawecki, T. J. (2010). Dietary restriction affects lifespan but not cognitive aging in Drosophila melanogaster. *Aging Cell*, *9*(3), 327–35. doi:10.1111/j.1474-9726.2010.00560

Carvalho, M., Schwudke, D., Sampaio, J. L., Palm, W., Riezman, I., Dey, G., … Eaton, S. (2010). Survival strategies of a sterol auxotroph. *Development (Cambridge, England)*, *137*(21), 3675–85. doi:10.1242/dev.044560

Drummond-Barbosa, D., & Spradling, a C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. *Developmental Biology*, *231*(1), 265–78. doi:10.1006/dbio.2000.0135

Dweck, H. K. M., Ebrahim, S. a M., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., … Stensmyr, M. C. (2013). Olfactory preference for egg laying on citrus substrates in Drosophila. *Current Biology : CB*, *23*(24), 2472–80. doi:10.1016/j.cub.2013.10.047

Emlen, D. J. (1994). Environmental Control of Horn Length Dimorphism in the Beetle Onthophagus acuminatus (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London. Series B: Biological Sciences* , *256* (1346), 131–136. doi:10.1098/rspb.1994.0060

Etges, W. J., & Heed, W. B. (1987). Sensitivity to larval density in populations of Drosophila mojavensis: Influences of host plant variation on components of fitness. *Oecologia*, *71*(3), 375–381. doi:10.1007/BF00378710

Fanson, B. G., & Taylor, P. W. (2012). Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios. *Age (Dordrecht, Netherlands)*, *34*(6), 1361–8. doi:10.1007/s11357-011-9308-3

Fanson, B. G., Weldon, C. W., Pérez-Staples, D., Simpson, S. J., & Taylor, P. W. (2009). Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (Bactrocera tryoni). *Aging Cell*, *8*(5), 514–23. doi:10.1111/j.1474-9726.2009.00497

Fellows, D. P., & Heed, W. B. (1972). Factors Affecting Host Plant Selection in Desert-Adapted Cactiphilic Drosophila. *Ecology*, *53*(5), 850–858. doi:10.2307/1934300

FITT, G. P. (1990). Variation in ovariole number and egg size of species of Dacus (Diptera; Tephritidae) and their relation to host specialization. *Ecological Entomology*, *15*(3), 255–264. doi:10.1111/j.1365-2311.1990.tb00807

Fogleman, J. C., & Danielson, P. B. (2001). Chemical interactions in the cactus-microorganism-Drosophila model system of the Sonoran Desert. *American Zoologist*, *41*(4), 877–889.

Gardiner, B. C. (1974). Observations on green pupae in Papilio machaon L. and Pieris brassicae L. *Wilhelm Roux' Archiv Für Entwicklungsmechanik Der Organismen*, *176*(1), 13–22. doi:10.1007/BF00577829

Gibert, J.-M., Peronnet, F., & Schlötterer, C. (2007). Phenotypic plasticity in Drosophila pigmentation caused by temperature sensitivity of a chromatin regulator network. *PLoS Genetics*, *3*(2), e30. doi:10.1371/journal.pgen.0030030

Godt, D., & Laski, F. a. (1995). Mechanisms of cell rearrangement and cell recruitment in Drosophila ovary morphogenesis and the requirement of bric à brac. *Development (Cambridge, England)*, *121*(1), 173–87.

Huang, X., Hancock, D. P., Gosby, A. K., McMahon, A. C., Solon, S. M. C., Le Couteur, D. G., … Simpson, S. J. (2013). Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity (Silver Spring, Md.)*, *21*(1), 85–92. doi:10.1002/oby.20007

Ja, W. W., Carvalho, G. B., Zid, B. M., Mak, E. M., Brummel, T., & Benzer, S. (2009). Water- and nutrient-dependent effects of dietary restriction on Drosophila lifespan. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(44), 18633–7. doi:10.1073/pnas.0908016106

Jaenike, J. (1978). On optimal oviposition behavior in phytophagous insects. *Theoretical Population Biology*, *14*(3), 350–356. doi:10.1016/0040-5809(78)90012-6

Jaenike, J., & Markow, T. a. (2003). Comparative elemental stoichiometry of ecologically diverse Drosophila. *Functional Ecology*, *17*(1), 115–120. doi:10.1046/j.1365-2435.2003.00701.x

Kambysellis MP, Heed WB. 1971 Studies of oogenesis in natural populations of Drosophilidae. I. Relation of ovarian development and ecological habitats of the Hawaiian species. Am. Nat. 941, 31 – 49. doi:10.1086/282700

Kennell, J. a, Cadigan, K. M., Shakhmantsir, I., & Waldron, E. J. (2012). The microRNA miR-8 is a positive regulator of pigmentation and eclosion in Drosophila. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *241*(1), 161–8. doi:10.1002/dvdy.23705

Kerkis, J. (1931). The Growth of the Gonads in DROSOPHILA MELANOGASTER. *Genetics*, *16*(3), 212–224.

King, R. C. (1970). *Ovarian Development in Drosophila melanogaster*. (227pp).

Klepsatel, P., Gáliková, M., De Maio, N., Ricci, S., Schlötterer, C., & Flatt, T. (2013). Reproductive and post-reproductive life history of wild-caught Drosophila melanogaster under laboratory conditions. *Journal of Evolutionary Biology*, *26*(7), 1508–1520. doi:10.1111/jeb.12155

Koyama, T., Mendes, C. C., & Mirth, C. K. (2013). Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. *Frontiers in Physiology*, *4*(September), 263. doi:10.3389/fphys.2013.00263

Krijger, C. L., Peters, Y. C., & Sevenster, J. G. (2001). Competitive ability of neotropical Drosophila predicted from larval development times. *Oikos*, *92*(2), 325–332. doi:10.1034/j.1600-0706.2001.920215.x

Kronforst, M. R., Barsh, G. S., Kopp, A., Mallet, J., Monteiro, A., Mullen, S. P., … Hoekstra, H. E. (2012). Unraveling the thread of nature's tapestry: the genetics of diversity and convergence in animal pigmentation. *Pigment Cell & Melanoma Research*, *25*(4), 411–33. doi:10.1111/j.1755-148X.2012.01014.x

Lachaise, D., Tsacas, L. & Couturier, G. (1982) The Drosophilidae associated with tropical African figs. Evolution 36, 141-151

Lang, M., Murat, S., Clark, A. G., Gouppil, G., Blais, C., Matzkin, L. M., … Orgogozo, V. (2012). Mutations in the neverland gene turned Drosophila pachea into an obligate specialist species. *Science (New York, N.Y.)*, *337*(6102), 1658–61. doi:10.1126/science.1224829

Leather, S. R., Wellings, P. W., & Walters, K. F. A. (1988). Variation in ovariole number within the Aphidoidea. *Journal of Natural History*, *22*(2), 381–393. doi:10.1080/00222938800770271

Lee, K. P., Behmer, S. T., Simpson, S. J., & Raubenheimer, D. (2002). A geometric analysis of nutrient regulation in the generalist caterpillar Spodoptera littoralis (Boisduval). *Journal of Insect Physiology*, *48*(6), 655–665.

Lee, K. P., Raubenheimer, D., Behmer, S. T., & Simpson, S. J. (2003). A correlation between macronutrient balancing and insect host-plant range: evidence from the specialist caterpillar Spodoptera exempta (Walker). *Journal of Insect Physiology*, *49*(12), 1161–1171. doi:10.1016/j.jinsphys.2003.08.013

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., … Raubenheimer, D. (2008). Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(7), 2498–503. doi:10.1073/pnas.0710787105

Lee, K. P., Simpson, S. J., & Raubenheimer, D. (2004). A comparison of nutrient regulation between solitarious and gregarious phases of the specialist caterpillar, Spodoptera exempta (Walker). *Journal of Insect Physiology*, *50*(12), 1171–80. doi:10.1016/j.jinsphys.2004.10.009

Lefranc A, Bundgaard J (2000) The influence of male and female body size on copulation duration and fecundity in Drosophila melanogaster. Hereditas 132:243–247

Markow, T. A., & O'Grady, P. M. (2005). EVOLUTIONARY GENETICS OF REPRODUCTIVE BEHAVIOR IN DROSOPHILA: Connecting the Dots. *Annual Review of Genetics*, *39*(1), 263–291. doi:10.1146/annurev.genet.39.073003.112454

Markow, T. a., Raphael, B., Dobberfuhl, D., Breitmeyer, C. M., Elser, J. J., & Pfeiler, E. (1999). Elemental stoichiometry of Drosophila and their hosts. *Functional Ecology*, *13*(1), 78–84. doi:10.1046/j.1365-2435.1999.00285.x

Matzkin, L. M., Johnson, S., Paight, C., Bozinovic, G., & Markow, T. a. (2011). Dietary protein and sugar differentially affect development and metabolic pools in ecologically diverse Drosophila. *The Journal of Nutrition*, *141*(6), 1127–33. doi:10.3945/jn.111.138438

Mayntz, D., Raubenheimer, D., Salomon, M., Toft, S., & Simpson, S. J. (2005). Nutrient-specific foraging in invertebrate predators. *Science (New York, N.Y.)*, *307*(5706), 111–3. doi:10.1126/science.1105493

Min, K.-J., Flatt, T., Kulaots, I., & Tatar, M. (2007). Counting calories in Drosophila diet restriction. *Experimental Gerontology*, *42*(3), 247–51. doi:10.1016/j.exger .2006.10.009

Mirth, C. K., & Riddiford, L. M. (2007). Size assessment and growth control: how adult size is determined in insects. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology*, *29*(4), 344–55. doi:10.1002/bies.20552

Mirth, C. K., & Shingleton, A. W. (2012). Integrating body and organ size in Drosophila: recent advances and outstanding problems. *Frontiers in Endocrinology*, *3*(April), 49. doi:10.3389/fendo.2012.00049

Mirth, C., Truman, J. W., & Riddiford, L. M. (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. *Current Biology : CB*, *15*(20), 1796–807. doi:10.1016/j.cub.2005.09.017

Nunney, L. (1990). Drosophila on Oranges: Colonization, Competition, and Coexistence. *Ecology*, *71*(5), 1904–1915. doi:10.2307/1937598

Partridge, L., Ewing, A., & Chandler, A. (1987). Male size and mating success in Drosophila melanogaster: the roles of male and female behaviour. *Animal Behaviour*, *35*(2), 555–562. doi:10.1016/S0003-3472(87)80281-6

Partridge, L., & Farquhar, M. (1983). Lifetime mating success of male fruitflies (Drosophila melanogaster) is related to their size. *Animal Behaviour*, *31*(3), 871–877. doi:10.1016/S0003-3472(83)80242-5

Partridge, L., Piper, M. D. W., & Mair, W. (2005). Dietary restriction in Drosophila. *Mechanisms of Ageing and Development*, *126*(9), 938–50. doi:10.1016/j.mad.2005.03.023

Raubenheimer, D., & Simpson, S. J. (2003). Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. *Journal of Experimental Biology*, *206*(10), 1669–1681. doi:10.1242/jeb.00336

Raubenheimer, D., & Jones, S. a. (2006). Nutritional imbalance in an extreme generalist omnivore: tolerance and recovery through complementary food selection. *Animal Behaviour*, *71*(6), 1253–1262. doi:10.1016/j.anbehav.2005.07.024

Raubenheimer, D., & Simpson, S. J. (1999). Integrating nutrition: a geometrical approach. *Entomologia Experimentalis et Applicata*, *91*(1), 67–82. doi:10.1046/j.1570-7458.1999.00467.x

Ribeiro, C., & Dickson, B. J. (2010). Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Current Biology : CB*, *20*(11), 1000–5. doi:10.1016/j.cub.2010.03.061

Rodrigues, M.A., Martins, N.E., Balancé, L.F., Broom, L.N., Dias, A.J.S., Fernandes, A.S.D., Rodrigues, F., Sucena, E., and Mirth, C.K.. Drosophila melanogaster larvae make foraging choices to minimize developmental time. In review.

Sahut-Barnola, I., Dastugue, B., & Couderc, J. L. (1996). Terminal filament cell organization in the larval ovary of Drosophila melanogaster: Ultrastructural observations and pattern of divisions. *Roux's Archives of Developmental Biology*, *205*(7-8), 356–363.

Sahut-Barnola, I., Godt, D., Laski, F. A., & Couderc, J. L. (1995). Drosophila ovary morphogenesis: analysis of terminal filament formation and identification of a gene required for this process. *Developmental Biology*, *170*(1), 127–35. doi:10.1006/dbio.1995.1201

Schiabor Allison S Eisen, Michael, K. M. Q. (2014). Non-respiratory functions of Saccharomyces cerevisiae mitochondria are required for optimal attractiveness to Drosophila melanogaster. *bioRxiv*.

Schwartz, N. U., Zhong, L., Bellemer, A., & Tracey, W. D. (2012). Egg laying decisions in Drosophila are consistent with foraging costs of larval progeny. *PloS One*, *7*(5), e37910. doi:10.1371/journal.pone.0037910

Schwarz, S., Durisko, Z., & Dukas, R. (2014). Food selection in larval fruit flies: dynamics and effects on larval development. *Die Naturwissenschaften*, *101*(1), 61–8. doi:10.1007/s00114-013-1129-z

Shakhmantsir, I., Massad, N. L., & Kennell, J. a. (2014). Regulation of cuticle pigmentation in drosophila by the nutrient sensing insulin and TOR signaling pathways. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *243*(3), 393–401. doi:10.1002/dvdy.24080

Simpson, S. J., Batley, R., & Raubenheimer∗, D. (2003). Geometric analysis of macronutrient intake in humans: the power of protein? *Appetite*, *41*(2), 123–140. doi:10.1016/S0195-6663(03)00049-7

Simpson, S. J., & Raubenheimer, D. (1993). A Multi-Level Analysis of Feeding Behaviour: The Geometry of Nutritional Decisions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *342*(1302), 381–402. doi:10.1098/rstb.1993.0166

Simpson, S. J., & Raubenheimer, D. (2005). Obesity: the protein leverage hypothesis. *Obesity Reviews : An Official Journal of the International Association for the Study of Obesity*, *6*(2), 133–42. doi:10.1111/j.1467-789X.2005.00178.x

Simpson, S. J., and Raubenheimer, D. (2012). 'The Nature of Nutrition: a Unifying Framework from Animal Adaptation to Human Obesity. (Princeton University Press: Princeton, NJ.)

Simpson, S. J., Raubenheimer, D., Behmer, S. T., Whitworth, a, & Wright, G. a. (2002). A comparison of nutritional regulation in solitarious- and gregarious-phase nymphs of the desert locust Schistocerca gregaria. *The Journal of Experimental Biology*, *205*(Pt 1), 121–9.

Sokoloff, A. (1966). Morphological Variation in Natural and Experimental Populations of Drosophila pseudoobscura and Drosophila persimilis. *Evolution*, *20*(1), 49–71.

Spieth, H. T. (1979). The Virilis Group of Drosophila and the Beaver Castor. *The America Naturalist*, *114*(2), 312–316.

Stone, W. S., Guest, W. C., & Wilson, F. D. (1960). . THE EVOLUTIONARY IMPLICATIONS OF THE CYTOLOGICAL POLYMORPHISM AND PHYLOGENY OF THE VIRILIS GROUP OF DROSOPHILA. *Proceedings of the National Academy of Sciences*, *46*(3), 350–361. doi:10.1073/pnas.46.3.350

Throckmorton, L. H. (1977). Drosophila Systematics and Biochemical Evolution. *Annual Review of Ecology and Systematics*, *8*(1), 235–254. doi:10.1146/annurev.es.08.110177.001315

Throckmorton, L.H. (1982) The virilis species group. In The Genetics and Biology of Drosophila, Vol. 3b (Ashburner, M., Carson, H.L. and Thompson, J.N. Jr., eds), pp. 227–296. Academic Press, London

Trumper, S., & Simpson, S. J. (1993). Regulation of salt intake by nymphs of Locusta migratoria. *Journal of Insect Physiology*, *39*(10), 857–864. doi:10.1016/0022-1910(93)90118-B

Zhou, H., Cadigan, K. M., & Thiele, D. J. (2003). A copper-regulated transporter required for copper acquisition, pigmentation, and specific stages of development in Drosophila melanogaster. *The Journal of Biological Chemistry*, *278*(48), 48210–8. doi:10.1074/jbc.M309820200

Zucoloto, F. S. (1987). Feeding habits of Ceratitis capitata (Diptera: Tephritidae): Can larvae recognize a nutritionally effective diet? *Journal of Insect Physiology*, *33*(5), 349–353. doi:10.1016/0022-1910(87)90123-5

SUPPLEMENTARY INFORMATION

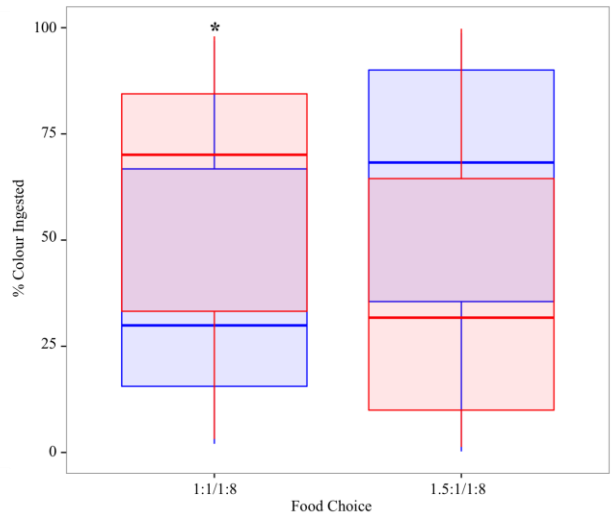

Figure S1 – Larvae show a preference for red when offered a choice between 1:1 and 1:8 but do not have a colour preference in the 1.5:1 and 1:4 choice. As a control for possible colour preference, we quantified the total percentage of each dye (red and blue) ingested by larvae for each food choice offered by spectrophotometer. Asterisks represents the Wilcoxon signed rank test V=609 and p-value=0.02451 for red colour preference (*p<0.05, **p<0.01, ***p<0.001).

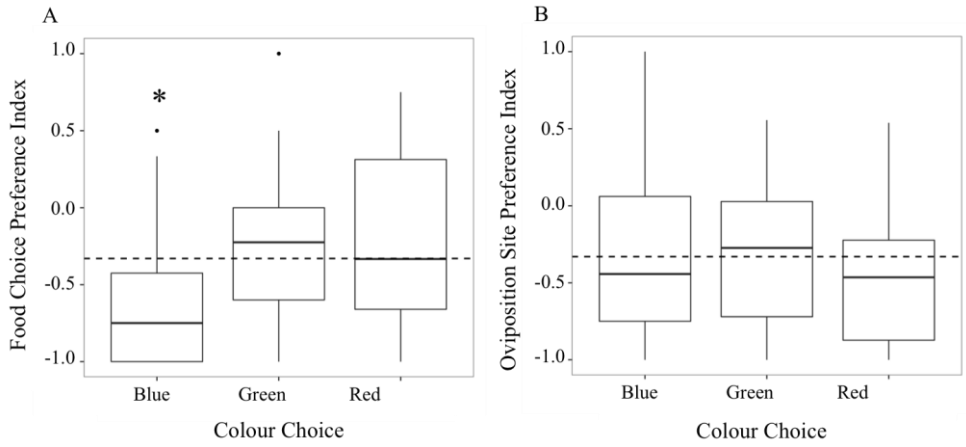

Figure S2 – Female Colour choice for food and oviposition preference. (A) Female preference index when offered Blue, Green and Red colours. Preference index was calculated by (# females that ate Blue - # females that ate Green - # females that ate Red) / (total # females - # females that did not eat - # number of females that ate more than one colour). (B) Female oviposition colour preference index (preferred colour to lay their eggs) when offered Blue, Green and Red colours. Preference index for oviposition colour site was calculated by (# eggs in Blue - # eggs in Green - # eggs in Red) / (total # eggs laid). In both plots the dashed line represents the no-choice value (- 0.33). The asterisks indicate significant difference to the no-choice value (-0.33).

Table S1: Female food and oviposition colour choice. The table represents a Wilcoxon signed rank test for each colour offered. Significant values are shown in bold. The significant interactions are highlighted in bold (*p<0.05, **p<0.01, ***p<0.001).

Female Colour Choice			
	Blue	Green	Red
V	**48**	158	148
p-value	**0.01087 ***	0.3134	0.4945
Oviposition Colour Choice			
	Blue	Green	Red
V	131	124	88
p-value	0.8967	0.9482	0.2172

Table S2 – Percentage and absolute number of females analysed (Total Females), females that did not choose any P:C ratio (No Choice), females that ate more than one P:C ratio(Mix Choice) and females that actually chose one of the P:C ratios offered (Choice). Only the females that chose one of the P:C ratios offered were used for the food choice preference index.

Total Females	No Choice	Mix Choice	Choice
473	325	3	145
100%	68.7%	0.6%	30.7%